Fetal development after antenatal exposures: chorioamnionitis and maternal glucocorticoids

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CHAPTER 1

General introduction

Preterm birth

Preterm birth is defined by the World Health Organization (WHO) as birth before the completion of 37 weeks of gestation (1, 2). Up to 13% of babies are born preterm with the incidence rising due to delayed childbearing and the use of assisted reproductive technologies which often results in multiple pregnancies (3, 4). Despite the advances in medical care, preterm birth still account for up to 75% of all perinatal mortality and morbidity in the Western world (2, 5).

In the last decades, administration of maternal glucocorticoids and surfactant replacement therapy has led to increased survival of preterm babies. It is now recommended that maternal glucocorticoids should be administered to women who are at risk of preterm labor before 34 weeks of gestation by use of a single course of either betamethasone (two doses of 12 mg intra-muscular, 24 hours apart) or dexamethasone (four doses of 6 mg intra-muscular, 12 hours apart) (6). Administration of these antenatal glucocorticoids enhances fetal lung maturation and thereby reduces the risk of death, respiratory distress syndrome, intraventricular hemorrhage and necrotizing enterocolitis in preterm neonates (6). It remains uncertain if the effects of maternal glucocorticoids on the fetal lung are only transient. Therefore, many clinicians choose to administer repeated courses of antenatal glucocorticoids to women who remain at risk of early labor (7, 8). However, concerns have risen about the effect of antenatal glucocorticoids on fetal growth, the immune system and neurological development especially when multiple courses are administered over gestation (9, 10).

Inflammation as a cause of preterm birth

Around 30% of preterm births are classified as indicated preterm birth in case the fetus is delivered preterm by caesarean section when the health of the mother or the fetus is at risk (2). The majority of preterm births are however considered as spontaneous preterm birth which can be initiated by various biological events, including maternal stress, placental dysfunction and infection or inflammation (2).

Chorioamnionitis, an intra-amniotic inflammation of the placental membranes and amniotic fluid, is one of the major causes of spontaneous preterm birth (11, 12). The incidence of chorioamnionitis has been reported to be up to 60% in very low birth weight (VLBW) infants (11, 13). Chorioamnionitis is commonly associated with pathogens of low virulence such as *Ureaplasma* and *Mycoplasma* species (14, 15). Based on the clinical course, chorioamnionitis can be subdivided into clinical chorioamnionitis and histological chorioamnionitis. Clinical chorioamnionitis can manifest with maternal fever, uterine tenderness and maternal and fetal tachycardia (13, 16). However, in the majority of cases, chorioamnionitis is a clinically silent infection without maternal symptoms in which case it is referred to as histological chorioamnionitis (11). Histological chorioamnionitis can only be diagnosed after birth by

histological examination of the placenta and fetal membranes (16). If chorioamnionitis is present, the fetus can be exposed to the micro-organisms or maternal pro-inflammatory cytokines through direct contact with the contaminated amniotic fluid or indirectly through the placental–fetal circulation (17). The exposure of the fetal epithelium of the skin, gut and lung may cause a systemic inflammatory response (FIRS) which is associated with adverse effects on the fetal lungs, brain, eyes, thyroid, heart, gut, liver, thymus and the immune system (17-19).

Chorioamnionitis as multi-organ disease of the fetus

Chorioamnionitis is associated with adverse neonatal outcomes in preterm infants (17, 20). Research mainly focused on the acute respiratory and neurological complications after exposure to intra-uterine inflammation (16). Recent data however suggest additional fetal sequelae of chorioamnionitis in various other organ systems such as the intestinal tract and the immunological organs (17, 21, 22). It is now clear that the effects of chorioamnionitis on the development and health of the fetus extend beyond the neonatal period and can have a long term impact on postnatal life. As many fetal organs may be affected by exposure to *in utero* inflammation, the concept of chorioamnionitis as a multi-organ disease of the fetus has emerged (Figure 1).

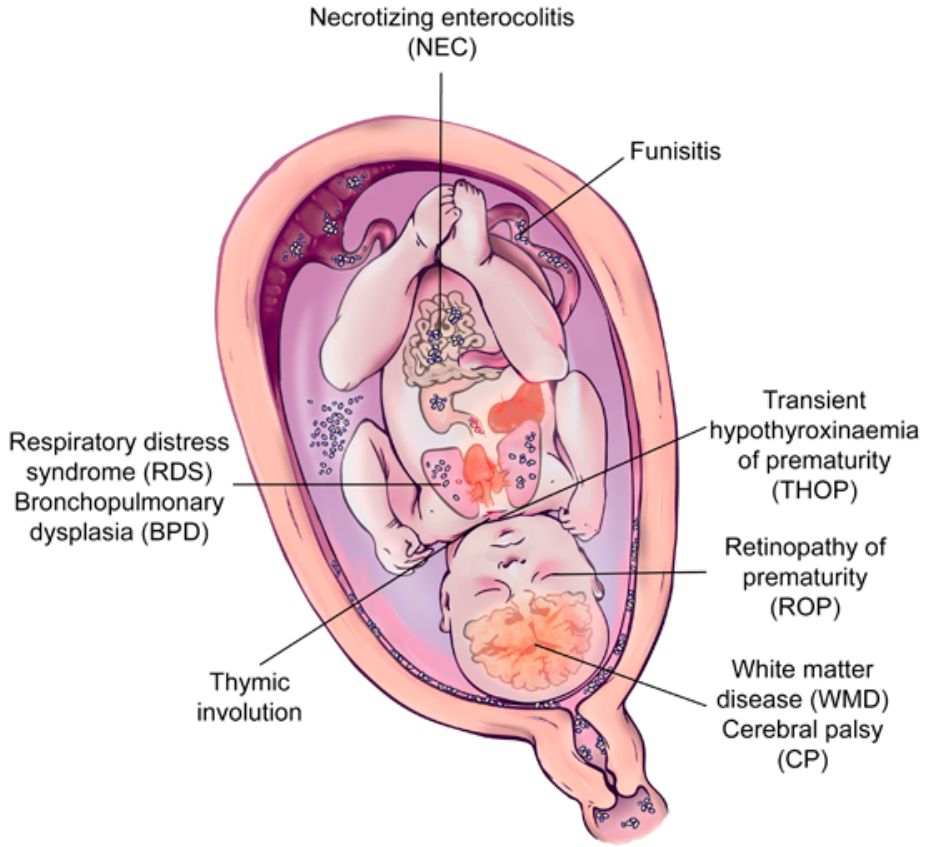

Figure 1: Complications in preterm infants related to the presence of chorioamnionitis during gestation (17).

As maternal glucocorticoids are highly effective in reducing neonatal mortality and morbidity in preterm infants, is has become standard of care in case of imminent preterm delivery irrespective of the cause of preterm birth (23). Because chorioamnionitis is often clinically silent prior to preterm labor, the combined exposures to antenatal glucocorticoids and chorioamnionitis are very common in preterm fetuses. However, there is a general lack of knowledge concerning the interactive effects of glucocorticoids and chorioamnionitis on fetal development and outcomes after preterm birth. The controlled situation of an experimental animal model may therefore provide valuable information in understanding neonatal outcomes after complex fetal exposures.

The ovine fetus as a translational animal model of chorioamnionitis

In the last years translational research with various animal models has been helpful to answer some basic questions about the effect of various fetal exposures on different organs. Rodent (24, 25) as well as rabbit (26), guinea pig (27) and sheep (28, 29) models have been used to study the effects of pre- and postnatal exposures on fetal development. However, the various animal models differ in their developmental biology compared to humans (30).

Development of the ovine immature brain, lung and gut are highly similar to that of humans (Figure 2). In terms of white matter maturation, myelination of the preterm human and ovine brain commences prenatally, whereas rodent white matter maturation predominantly takes place after birth (31). Similarly, the alveolar phase of lung development starts prenatally in humans and sheep but postnatally in rodents (30). In addition, the long gestational period in sheep (~147 days) allows fetal development to be studied at specific time-points and over longer periods of time. Therefore preterm sheep are considered as an excellent translational model to study the effects of antenatal events on the developing fetus.

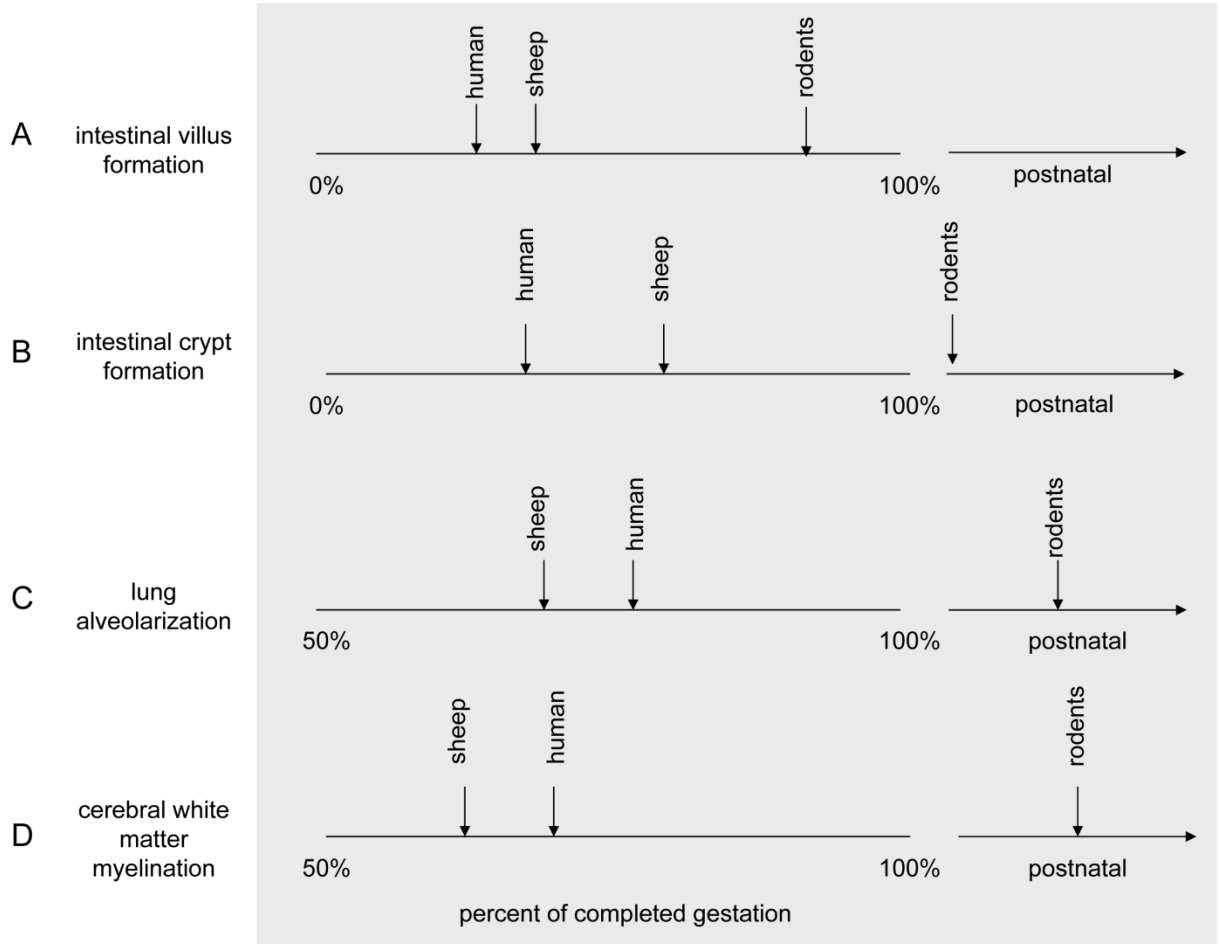

Figure 2: Comparison of important hallmarks in fetal gut (A, B), lung (C), and brain (D) development over gestation in humans, sheep and rodents (21).

Antenatal exposures and fetal lung development

Exposure to chorioamnionitis over gestation can have adverse effects on fetal lung development (18, 32). The infectious micro-organisms in the amniotic fluid can invade the respiratory system of the fetus and induce inflammation in the fetal lung (33). This inflammatory response damages the immature lungs and influences lung development by accelerating lung maturation (34). However, by inducing early lung maturation, late lung development is impaired (35). During the last phase of lung development which is called the alveolarization phase, functional alveoli are formed by secondary septation which subsequently increases the surface area needed for optimal gas exchange (36). In this phase, the existing airspaces are subdivided into smaller alveoli, by the outgrowth of secondary septa. This process is orchestrated by interactions between myofibroblasts, extracellular matrix proteins, the vascular endothelial cells and the airway epithelial cells. Pulmonary myofibroblasts produce focal deposits of elastin, an extracellular matrix component which functions as a structural navigator for the formation of the growing alveolus (36). Inhibition of late lung development by intra-uterine exposure to inflammation, will lead to a decreased

number of alveoli and less surface area for gas exchange which eventually impairs the lung function of the newborn.

Not only exposure to pro-inflammatory stimuli is a risk factor for adverse fetal lung development. Although antenatal glucocorticoids enhance lung maturation by increasing the surfactant production and lung compliance which improves neonatal outcome, glucocorticoids also interfere with the process of fetal lung development (37, 38). As a result, a large number of preterm infants are exposed *in utero* to various stimuli which can disrupt late lung development. At birth, the lungs of these preterm babies may have larger and fewer alveoli, disordered elastin depositions and impaired pulmonary microvasculature development. This altered lung morphology can form the basis for bronchopulmonary dysplasia (BPD) (39). BPD is the most common chronic lung disease in preterm infants and is defined clinically as a need for additional oxygen support at 36 weeks postmenstrual age (40). Apart from intensive hospital care in early life, BPD infants also have an increased risk for recurrent respiratory complications such as wheezing and respiratory infections, and neurodevelopmental disabilities (41, 42). To date no effective treatment is available for BPD as the basic molecular mechanisms by which inflammation and glucocorticoids may disturb normal fetal lung development remain unclear.

Several signaling cascades and growth factors are essential in directing pulmonary development in the fetus. The Wingless-Int (Wnt) signaling pathway is involved in both early and late stages of lung development by regulation of branching and epithelial and mesenchymal interactions (43, 44). Dysregulation of Wnt signaling interrupts the process of lung branching and maturation as demonstrated in several knock out models. Wnt2-/- mice have severe lung hypoplasia and a poorly developed lung mesenchyme at birth (45). Knockout of Wnt7b in mice also results in lung hypoplasia and perinatal death due to respiratory failure (46, 47). The Sonic Hedgehog (Shh) signaling cascade is a second pathway critical for lung development as Shh-null mice have hypoplastic lungs and die due to respiratory failure (48). The Shh pathway regulates the expression of lung growth factors such as fibroblast growth factor 10 (FGF10) and bone morphogenetic protein 4 (BMP4) which both mediate branching and myofibroblast differentiation (49, 50). As both Wnt and Shh signaling are essential in fetal lung development, dysregulation of these pathways by antenatal exposures could potentially result in disrupted lung morphology as seen in BPD patients.

Antenatal exposures and the fetal immunological organs

Based on Kunzmann et al.(18)

Preterm infants are highly susceptible to postnatal infections and sepsis due to the immaturity of the neonatal immune system (21, 51). It appears however that various prenatal factors, including intra-uterine exposure to inflammation, can alter the maturity and function of the immune system already *in utero* (52, 53). Several clinical studies observed involution of both the thymus and spleen in preterm infants born after intra-amniotic exposure to inflammation (54-56). In a histological study on the immunological organs of fetuses with severe clinical chorioamnionitis, splenic and thymic depletion with histological features of degenerative processes were described (54, 57). These immunological changes induced by prenatal inflammation could impose a serious disadvantage for preterm infants as it may predispose them for other adverse neonatal outcomes (58, 59). A small thymus detected at birth by routine chest radiograph is predictive for the development of BPD in very low birth weight preterm infants (60). Thymic involution in preterm infants is associated with a higher risk for the development of cerebral white matter damage, the major antecedent of cerebral palsy (61). Thymic involution correlates with the frequency of infections in preterm infants admitted to the neonatal intensive care unit, with the grade of thymic involution being related to the duration of the illness (62). Moreover, a small thymus size at birth can lead to a smaller T-cell repertoire and lower thymic output leading to enhanced vulnerability to infections in later life (63, 64).

As the immune system is an important target for glucocorticoids, concerns have risen about the effects of antenatal glucocorticoid treatment on the development and function of the fetal immune system (65). Early clinical reports demonstrated that the incidence of hospital admissions due to infectious diseases in the first year of life was significantly higher in preterm infants exposed to antenatal glucocorticoids compared to preterms that were not treated with glucocorticoids (66). Furthermore, Chabra et al. showed a reduced lymphocyte count in the cord blood of preterms exposed to antenatal glucocorticoids which could contribute to the increased susceptibility for postnatal infections (67). Studies in experimental animal models demonstrated that antenatal glucocorticoids induced thymic involution and decreased T-cell numbers in the fetal thymus and spleen (68). Immune function was also compromised with reduced T-cell and B-cell reactivity, decreased T-cell proliferation and suppression of monocyte function (69-71). These changes may impair the functional maturity of the neonatal immune system and could contribute to the increased incidence and adverse outcome of postnatal infections. Despite the growing evidence that both exposure to intra-uterine inflammation and maternal glucocorticoids has profound effects on the fetal immunological organs (52, 53, 71), little is known about the interactive effects of both prenatal exposures on the fetal thymus and spleen, and the consequences of these changes on the function of the immune system in later life.

Antenatal exposures and fetal brain development

Based on Kuypers et al.(72)

Preterm birth and chorioamnionitis are both major risk factors for perinatal brain damage and neurodevelopmental complications (16, 73). The presence of chorioamnionitis is related to the development of cerebral white matter damage (WMD) which in severe cases presents clinically as cerebral palsy; a syndrome characterized by motoric and cognitive deficits (74-76). In addition, exposure to inflammation during gestation increases the risk for neurological complications such as intraventricular hemorrhage, thereby contributing to perinatal morbidity and mortality (20, 77). Moreover, recent data suggest that chorioamnionitis is linked to an increased risk for other adverse neurological outcomes such as autism spectrum disorders (78, 79), schizophrenia (80) and cognitive impairments (81, 82). Pinelli et al. reported chorioamnionitis as an independent risk factor for positive autism screening in a 2 year follow up study of preterm infants (78) which was confirmed in a second cohort study from Limperopoulos et al. (79). Further neurodevelopmental assessments at 18 months of age showed that children exposed to clinical chorioamnionitis displayed lower cognitive language and motor scores compared to ex-preterms which were not exposed to chorioamnionitis (82, 83). Although several reports show similar conclusions with reduced mental developmental scores after clinical chorioamnionitis (84, 85), other studies point out that exposure to histological chorioamnionitis is not related to neurocognitive impairments in later life (86-88). These inconsistencies of various reports demonstrate that chorioamnionitis can present itself as a very variable condition from a mild, tolerable to a severe, exuberant infection, subsequently causing fetal brain injury.

Intra-uterine exposure to infection/inflammation induces a rapid increase of pro-inflammatory cytokines in the amniotic fluid (89, 90), fetal serum (91, 92) and in the fetal central nervous system (92, 93). Pro-inflammatory cytokines can cause direct damage to the developing brain by inducing apoptosis and disrupting the differentiation of neurons and oligodendrocytes (94, 95). Moreover, pro-inflammatory cytokines can also induce indirect damage by activation of microglia, the resident immune cells of the brain (96). Microglia survey the microenvironment by movement of their ramified processes and can rapidly be activated by various alterations in the brain parenchyma (97). Upon activation, microglia change their morphology and proliferate and migrate to the site of action where they can secrete pro-inflammatory cytokines, free radicals and excitatory amino acids (98-100). A growing body of evidence supports the concept that microglia activation plays a central role in the pathogenesis of fetal brain injury after exposure to intra-uterine inflammation. Microglial cell activation has been detected in periventricular lesions of infants with periventricular leukomalacia (101, 102). Similarly, models of lipopolysaccharide-induced chorioamnionitis showed profound microglial activation in the fetal brain with loss of oligodendrocytes, the myelin producing cells of the brain, and consequent development of white matter disease (103, 104).

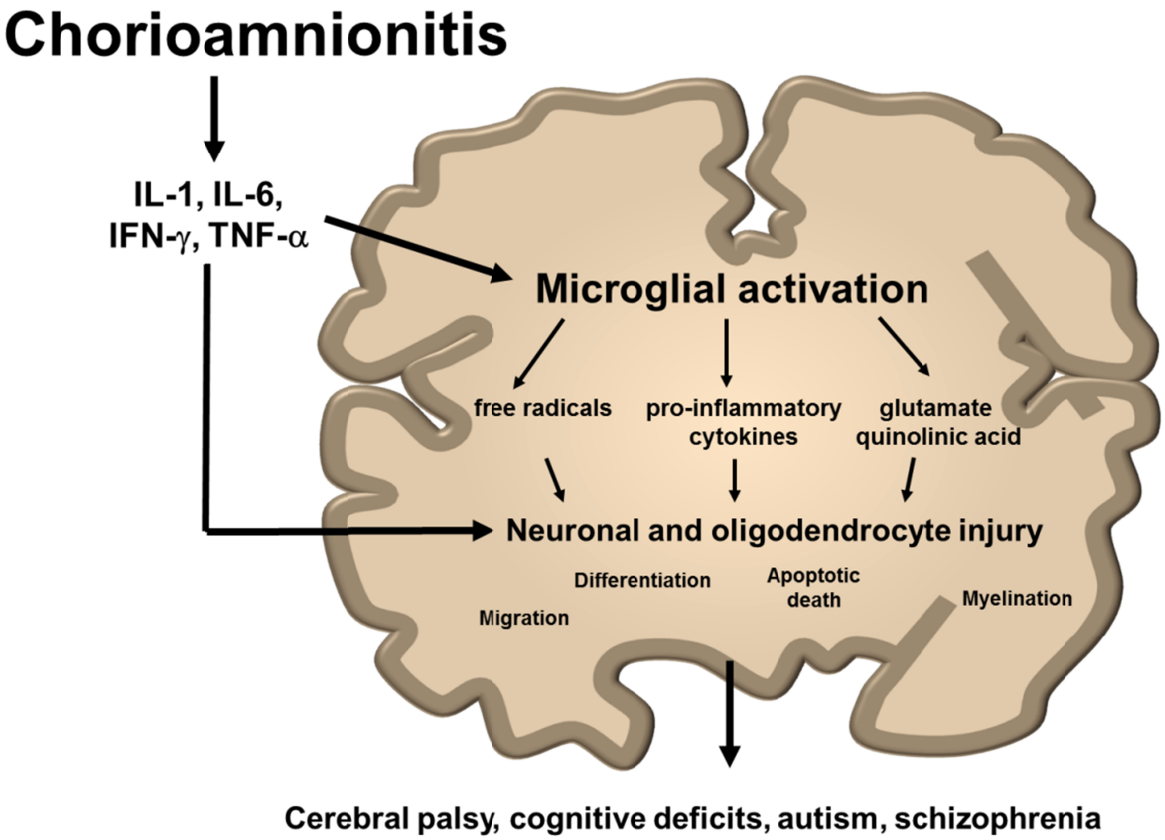

Figure 3: Proposed mechanisms of fetal brain injury after exposure to chorioamnionitis. Chorioamnionitis can induce an increase in pro-inflammatory cytokines in the fetus. These cytokines cause direct neuronal and oligodendrocyte injury or indirect damage by activation of microglia. Activated microglia secrete pro-inflammatory cytokines, free radicals and excitatory amino acids which injure the immature fetal neurons and oligodendrocytes resulting in apoptotic cell death, impaired differentiation and migration and loss of myelination. Therefore, microglial activation and oligodendrocyte damage after exposure to intra-uterine inflammation play a central role in the pathogenesis of cerebral palsy, cognitive deficits and psychiatric illnesses (72).

Antenatal glucocorticoid treatment can also influence the fetal brain. Aside from their beneficial effects on neonatal lung function, glucocorticoids improve neurological outcomes as they reduce the risk of intraventricular hemorrhage and periventricular leukomalacia in preterm infants (105, 106). These neurological benefits extend into later life with a decreased incidence of cerebral palsy and improved cognitive abilities (107, 108). However, evidence from experimental animal models show that antenatal glucocorticoids can also have adverse effects on the immature brain. In preterm lambs, repeated doses of maternal glucocorticoids reduced fetal brain weight and decreased myelination of the optic nerve and corpus callosum (109, 110). Furthermore, glucocorticoids reduced cell proliferation, synaptic density and the expression of cytoskeletal proteins in the fetal hippocampus (111-113). The discrepancies between human and experimental animal data do raise concerns about the possible harmful effects of antenatal glucocorticoids on fetal neurodevelopment especially in the case of combined exposure to intra-uterine inflammation.

Outline of thesis

Although the combined exposure to maternal glucocorticoids and chorioamnionitis are very common in preterm fetuses, little is known about the interactive effects of both antenatal factors on fetal development. Therefore, the general aim of this thesis was to study the combined effects of intra-uterine exposure to inflammation and antenatal glucocorticoids on the fetal lung, immune system and brain under controlled conditions of a translational animal model in order to better understand how these common prenatal events contribute to disease processes in postnatal life. For this purpose, a preterm lamb model was used as their development *in utero* is highly similar to human development.

In **chapter 2** we studied the effects of combined exposure to intra-uterine inflammation and maternal antenatal betamethasone on fetal lung inflammation and maturation. To gain a better understanding of the molecular mechanisms by which antenatal inflammation and glucocorticoids can modulate fetal lung development, we determined possible changes in pulmonary Shh and Wnt signaling, the molecular pathways important in directing pulmonary development, in **chapter 3** and **4**.

Although the systemic inflammatory response of the fetus after intra-uterine inflammation has been the focus of various studies, the effects on the fetal immunological organs has barely been investigated. Therefore we assessed the response of the fetal thymus to exposure to intra-uterine inflammation and antenatal glucocorticoids in **chapter 5 and 6.** In **chapter 7,** we showed that not only the thymus but also the fetal spleen is able to mount an immune response to *in utero* inflammation.

Apart from the adverse effects on lung development, antenatal inflammation can also affect the fetal brain. The effects of maternal glucocorticoid administration in this aspect are not well understood. To determine the neurodevelopmental effects of glucocorticoids in the settings of chorioamnionitis, we investigated fetal brain inflammation and injury after combined antenatal exposures in **chapter 8**. The major findings of this thesis and the implications for future research are discussed in **chapter 9**.

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CHAPTER 2

Intra-amniotic LPS and antenatal betamethasone: inflammation and maturation in preterm lamb lungs

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Abstract

The pro-inflammatory stimulus of chorioamnionitis is commonly associated with preterm delivery. Women at risk of preterm delivery receive antenatal glucocorticoids to functionally mature the fetal lung. However, the effects of the combined exposures of chorioamnionitis and antenatal glucocorticoids on the fetus are poorly understood. Time-mated ewes with singleton fetuses received an intra-amniotic injection of lipopolysaccharide (LPS) either preceding or following maternal intra-muscular betamethasone 7 or 14 days before delivery, and the fetuses were delivered at 120 days gestational age (GA) (term=150 days GA). Gestation matched controls received intra-amniotic and maternal intramuscular saline. In comparison with saline controls, intra-amniotic LPS increased inflammatory cells in the bronchoalveolar lavage and myeloperoxidase (MPO), Toll-like receptor 2 and 4 mRNA, PU.1, CD3, and Foxp3-positive cells in the fetal lung. LPS induced lung maturation measured as increased airway surfactant and improved lung gas volumes. Intra-amniotic LPS induced inflammation persisted until 14 days after exposure. Betamethasone treatment alone induced modest lung maturation, but when administered before intra-amniotic LPS, suppressed lung inflammation. Interestingly, betamethasone treatment after LPS did not counteract inflammation but enhanced lung maturation. We conclude that the order of exposures of intra-amniotic LPS or maternal betamethasone had large effects on fetal lung inflammation and maturation.

Introduction

Chorioamnionitis, defined as inflammation of the fetal membranes, complicates up to 70% of preterm deliveries before 30 weeks of gestation (16). The epidemiological associations of preterm infants exposed to chorioamnionitis are fetal systemic inflammation and lung, brain and gastrointestinal injury (17, 43). Lung inflammation may initiate a progressive injury that results in bronchopulmonary dysplasia (26). However, the exposure of the fetal lung to inflammation associated with chorioamnionitis also increases surfactant proteins and lipids with salutary effects on respiratory distress syndrome (2, 30, 37).

Antenatal corticosteroids are a standard treatment given to mothers at risk of imminent preterm birth to induce lung maturation (42). Lung maturation induced by corticosteroids results from the simultaneous induction and suppression of multiple genes that have the net effects of increasing surfactant lipids, surfactant proteins and thinning of the lung mesenchyme to increase the potential airspaces (23, 47, 49). Clinical studies and experimental evidence suggest that antenatal corticosteroids are also efficacious in the setting of chorioamnionitis (18, 40, 41). The delivery of many women given corticosteroids is delayed for several days to weeks (6), and chorioamnionitis is clinically silent in a majority of women (2). Therefore, the combined exposures of antenatal corticosteroids and chorioamnionitis are common in the preterm fetus, but the order of exposures can vary.

We reported previously that simultaneous exposure of the preterm sheep fetus to antenatal corticosteroids and lipopolysaccharide (LPS) suppressed the pulmonary inflammation for 1 to 2 days after the exposure but amplified the inflammatory response to chorioamnionitis 5 to 15 days after the exposure (28, 34). However, it is not known if the order of exposure is important in mediating lung inflammation and maturation. Therefore, we aimed to study the interactive effects of chorioamnionitis and antenatal corticosteroids on the inflammatory and maturational response in the fetal lungs in a clinically relevant preterm sheep model of chorioamnionitis (32). We tested the hypothesis that the order of exposure to maternal betamethasone and intra-amniotic LPS will differentially impact lung surfactants and inflammatory responses in the preterm fetus. Fetal sheep were exposed *in utero* to intra-amniotic LPS or antenatal maternal intra-muscular betamethasone, with an interval of 7 days between the two interventions. Furthermore, we asked if the order of exposure to antenatal corticosteroids and intra-amniotic LPS altered fetal lung outcomes.

Materials and methods

Animal model and sampling protocol

All studies were approved by the Animal Ethics Committees at The University of Western Australia and Cincinnati Children`s Hospital Medical Center. Time-mated ewes with singleton fetuses were randomly assigned to one of six treatment groups to receive an intra-amniotic (IA) injection of lipopolysaccharide (LPS) (10 mg *Escherichia Coli* 055:B5, Sigma Chemical, St.

Louis, MO, USA), intra-muscular (IM) injection of betamethasone (Beta) (Celestone Soluspan, Schering-Plough, North Ryde, New South Wales (NSW), Australia, 0.5 mg/kg maternal weight) or an equivalent injection of saline (controls) at 107 days and/or 114 days GA in different permutations and combinations (Figure 1). All ewes in this study received a single intra-muscular injection of 150 mg medroxyprogesterone acetate (Depo-Provera, Kenral, NSW, Australia) at 100 days GA to prevent preterm birth induced by betamethasone treatment (24). Lambs were surgically delivered at 120 days GA (term = 150 days GA) and euthanized with 100 mg/kg pentobarbital. The fetus was weighed and fetal cord blood was collected. Following opening of the chest, a deflation air pressure-volume curve was measured from static inflation of the lung to 40 cm H2O airway pressure (25). The lungs were removed, separated and weighed prior to a bronchoalveolar lavage of the left lung with 0.9% NaCl (25). The bronchoalveolar lavage fluid (BALF) was used for differential cell counts and surfactant measurements. Lung tissue from the right lower lobe (RLL) was snap frozen and the right upper lobe (RUL) was inflation-fixed in 10% buffered formalin for 24 hours.

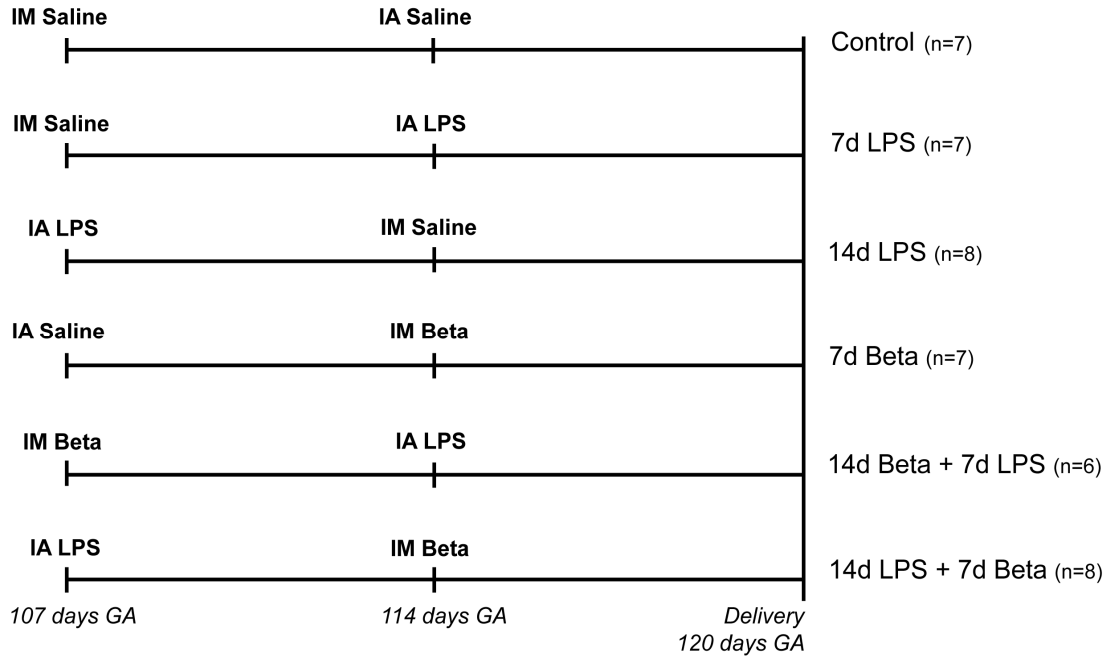

Figure 1: Study design: pregnant ewes received an intra-amniotic injection of lipopolysaccharide (LPS) and/or an intra-muscular injection of betamethasone (Beta) and/or an equivalent injection of saline for control animals at 107 days and/or 114 days gestation (GA). Lambs were delivered preterm by cesarean section at 120 days GA (term = 150 days GA).

Surfactant proteins and cytokine mRNA quantitation

Total RNA was isolated from frozen lung tissue of the RLL using a modified Chomzynski method and mRNA quantitation was performed using real-time PCR (27). Gene expression was measured for surfactant proteins and cytokines: SP-A, SP-B, SP-C, IL-1β, IL-6, IL-8, CP-1 and Serum amyloid A3. The mRNA was reverse transcribed to yield a single-strand cDNA (verso cDNA kit, Thermoscientific, UK), which was used as a template with primers and

Taqman probes (Applied Biosystems, Carlsbad CA, USA) specific to sheep sequences (29). The values for each cytokine were normalized to the internal 18S rRNA. Data were expressed as fold increased over control values.

Toll-like receptor mRNA quantitation

For the Toll like receptor (TLR) mRNA measurements, total RNA was extracted from frozen lung tissue of the RLL using the SV Total RNA Isolation system (Z3100, Promega, Madison, USA) according to the manufacturer's instructions. Genomic DNA contamination was removed by treatment with RQ1 DNase (M610A, Promega) and the RNA was tested for the presence of genomic GAPDH. Briefly, PCR amplification for the detection of genomic DNA was performed with DNA Taq Polymerase (M124B, Promega) at 95 °C for 5 minutes followed by 40 cycles at 95 °C for 30 seconds, 55 °C for 45 seconds and 72 °C for 30 seconds. Total RNA was used as a template. PCR products were analyzed on 1.5 % agarose gels. Total RNA was reverse transcribed with the First Strand cDNA synthesis kit (4379012001, Roche-Applied, Mannheim, Germany) according to manufacturer`s instructions using anchored oligo-primers. Primers for real time PCR (RT-PCR) were constructed based on published ovine or bovine cDNA sequences (Table 1).

Table 1: Primers used for RT-PCR

Gene		Sequence (5`-3`)	Amplicon size	T^m	Accession code (RefSeq)
TLR2	Fw	GGCTGTAATCAGCGTGTTCA	160bp	64°C	NM_001048231.1
	Rv	GATCTCGTTGTCGGACAGGT			
TLR4	Fw	GAGAAGACTCAGAAAAGCCTTGCT	200bp	65°C	NM_001135930.1
	Rv	GCGGGTTGGTTTCTGCAT			
TLR9	Fw	CCCTGGAGAAGCTGGACAT	175bp	60°C	NM_001011555.1
	Rv	GACAGGTCCACGAAGAGCAG			

Dilution experiments were performed to ensure similar PCR amplification efficiency of the primers. RT-PCR reactions were performed in duplicate with the LightCycler 480 SYBR Green I Master mix (4707516001, Roche-Applied) on a LightCycler 480 Instrument according to the manufacturer`s instructions. RT-PCR results were normalized to cyclophilin A, a household gene, and mean fold changes in mRNA expression were calculated by the ΔΔCt-method (38).

Surfactant saturated phosphatidylcholine (Sat PC) and SP-D

SP-D in the BALF was measured with a sandwich ELISA using rabbit anti-ovine SP-D as coating antibody and guinea pig anti-ovine SP-D a secondary antibody (19, 20). Sat PC was isolated from the BALF using organic solvent extraction, osmium tetroxide and neutral alumina followed by a phosphorus measurement (7).

Immunohistology

The following antibodies were used to identify different cell types: CD3 – T cells, Myeloperoxidase – activated neutrophils and monocytes, PU.1 – maturation marker for myeloid cells, Pro-Surfactant protein-C (SP-C) – maturation marker for alveolar type II cells, thyroid transcription factor-1 (TTF-1) – alveolar type II cells, Foxp3 – Regulatory T-cells. Paraffin embedded RUL lung sections (4 µm, transverse) were stained for CD3 (DAKO A0452, Dakocytomation, Glostrup, Denmark), myeloperoxidase (MPO) (DAKO A0398, Dakocytomation) PU.1 (sc-352, Santa Cruz Biotechnology, Santa Cruz, USA), Foxp3 (14-7979, eBioscience, San Diego, USA), thyroid transcription factor-1 (TTF-1) (WRAB-1231, Seven Hills Bioreagents, Cincinnati OH, USA) or Pro-SP-C (WRAB-9337, Seven Hills Bioreagents). Briefly, the sections were deparaffinized in an ethanol series and endogenous peroxidase-activity was blocked by incubation with 0.3 % H2O2 in 1x phosphate buffered saline (PBS, pH 7.4) or methanol (for TTF-1 and Pro-SP-C). Antigen retrieval was performed by incubating the sections in heated citrate buffer (10 mM, pH 6.0) for 30 minutes. In order to block nonspecific binding, the slides were incubated with 20 % normal goat serum (NGS) in PBS (for MPO and Foxp3), 5 % bovine serum albumin (BSA) in PBS (for CD3 and PU.1) or 2 % NGS in PBS (for TTF-1 and Pro-SP-C). Sections were incubated overnight at 4 °C with the diluted primary antibody (CD3 1:200, MPO 1:500, PU.1 1:400, Foxp3 1:30, TTF-1 1:100, Pro-SP-C 1:1500). After incubation with a goat-anti-mouse biotin labeled (for Foxp3) (DAKO E0433, Dakocytomation) or swine-anti rabbit biotin labeled secondary antibody (DAKO E0353, Dakocytomation), immunostaining was enhanced with Vectastain ABC peroxidase Elite kit (PK-6200, Vector Laboratories, Burlingame, USA) and stained with nickel sulfate-diaminobenzidine (NiDAB). Subsequently, the sections were rinsed in Tris/saline and incubated with Tris/cobalt. After counterstaining with 0.1 % Nuclear Fast Red, the sections were washed, dehydrated and cover-slipped.

Since the air-space expansion and tissue characteristics were different in the different groups, we measured tissue area in the lung sections. Blinded measurements of tissue fractions (expressed relative to the total lung area) were performed in 5 random non-overlapping fields (20x objective) for each animal and for at least 4 animals/group using the color threshold function of the program metamorph v6.1r0 (Molecular devices/Universal imaging corp., Sunnyvale CA). The average measurement from each lamb was used to compute a group average. The tissue fractions in different experimental groups were normalized to the control group average to obtain a correction factor. For example, if the tissue fraction in an experimental group was 1.2 times control, then the cells counted per microscopic field were divided by 1.2. Thus the expressed cell counts per microscopic field incorporated this correction factor.

Evaluation was performed by light microscopy (Axioskop 40, Zeiss, Germany) with LeicaQWin Pro v.3.4.0 software (Leica Microsystems, Germany). MPO-, CD3-, PU.1- and Pro-SP-C positive cells were counted in three representative high power fields at 200x magnification by a blinded observer and averaged per animal. Because FoxP3 positive cells were unevenly

distributed throughout the lung tissue, sections were scored for positive signal for Foxp3 with a semi-quantitative scoring system by a blinded observer: 1 – little staining, 2 – light staining, 3 – heavy staining. For TTF-1, a computer aided manual count of 5 random fields at 200x magnification per animal was performed, using the computer program metamorph (Image-Pro Plus v7.0). The MPO, CD3 and TTF-1 cell counts per microscopic field incorporated the correction factor for tissue fraction. The SP-C positive cells were expressed relative to TTF-1 positive cells to assess if changes were due to increased expression in each of the alveolar type II cell vs. increased numbers of alveolar type II cells in different groups.

Data Analysis

Results are given as means ± standard error of means (SEM). The groups were compared using one-way ANOVA with Tukey's test for post-hoc analysis as appropriate. Statistical analysis was performed by GraphPad Prism v5.0. Significance was accepted at $p<0.05$.

Results

Description of animals

The experiments were prospectively designed to test the interactions of antenatal corticosteroids and intra-amniotic LPS exposure. Despite the medroxyprogesterone acetate treatment, animals exposed to maternal betamethasone experienced fetal losses, such that we reassigned animals from the group which only received betamethasone 14 days before delivery to other treatment groups as our priority was to test the interaction of betamethasone and LPS. The intra-amniotic LPS injections were not associated with fetal losses, but fetal deaths identified by ultrasound and fetal losses were frequent in the betamethasone treated groups (Table 2). All animals had comparable birth weights except for a lower birth weight for animals with combined exposure to LPS 14 days and betamethasone 7 days before delivery. Cord blood pH values and lung to body weight ratios were comparable across all groups.

Table 2: Variables at birth

Treatment group	Fetal outcomes			Birth weight	Cord blood	Sex	Lung Wt/Body Wt
	Fetal death	Abortion	Alive	(kg)	pH	M/F	g/kg
Control	1	-	7	2.7±0.2	7.24±0.03	3/4	32.7±0.9
7d LPS	-	-	7	2.5±0.1	7.26±0.02	3/4	33.6±1.2
14d LPS	-	-	8	2.5±0.1	7.27±0.02	5/3	37.1±1.8
7d Beta	2	1	7	2.7±0.2	7.20±0.04	4/3	36.7±0.8
14d Beta + 7d LPS	-	3	6	2.7±0.1	7.25±0.04	1/5	36.2±2.4
14d LPS + 7d Beta	1	1	8	2.1±0.1*	7.25±0.03	4/4	39.9±2.7

Data expressed as mean ± SEM. * p<0.05 vs. controls using a one-way ANOVA with Tukey's post hoc test. LPS- Lipopolysaccharide, Beta- Betamethasone, GA- Gestational age, Wt- weight, M - male, F - female.

Lung inflammation

Pulmonary inflammation resulting from LPS and the anti-inflammatory effect of betamethasone exposures were assessed by differential cell counts on the BALF (Figure 2). Neutrophil levels were modestly increased 7 days after the exposure to LPS and significantly increased 14 days after LPS exposure (Figure 2A). This increase did not occur in lambs exposed to maternal betamethasone 7 days after intra-amniotic LPS. In this group, however, there was a 10-fold increase in monocytes, which was not seen in the groups exposed to LPS only (Figure 2B). Lymphocyte numbers did not differ in experimental groups compared to controls (Figure 2C). Lung inflammation was further characterized by identifying markers for activation of inflammatory cells (Figure 3). MPO-positive cells were significantly increased 14 days after LPS exposure (Figure 3A-D).

Betamethasone pre-treatment 7 days before the LPS exposure prevented the increase of MPO-positive cells in the fetal lung. However, betamethasone given 7 days after LPS only partially inhibited the influx of LPS-mediated MPO-positive cells. PU.1 expression, which is a transcription factor that indicates maturation of monocytes (35), increased 7 days after LPS exposure in the fetal lung (Figure 3E-H). This increase in PU.1 positive-cells could be prevented by pre-treatment with betamethasone 7 days before the LPS exposure. However, exposure to 14 days LPS followed by betamethasone for 7d increased PU.1 expressing cells in the lung. LPS exposure for either 7 or 14 days increased the number of CD3-positive cells in the fetal lung (Figure 3I).

The groups exposed to both betamethasone and LPS did not show a significant increase in CD3-positive cells compared to controls indicating that betamethasone pre- and post-treatment both countered the LPS-induced influx of CD3-positive cells. Foxp3-positive cells only increased in the lung tissue 7 days after LPS exposure (Figure 3J), which was inhibited by betamethasone pre-treatment. Consistent with our previous observation of maximal induction of cytokines 2 d after exposure (33, 36), the mRNA expression of *IL-1β*, *IL-6*, *IL-8* and *MCP-1* were only modestly increased 7 days after LPS exposure compared to controls (Table 3). Combined exposure to LPS and betamethasone had no significant effect on lung cytokine mRNA expression. The mRNA levels of Serum amyloid A, an acute phase reaction protein expressed in the lung and liver (50), increased 7 days after the LPS exposure compared to controls. Betamethasone pre-treatment prior to the LPS exposure suppressed this increase.

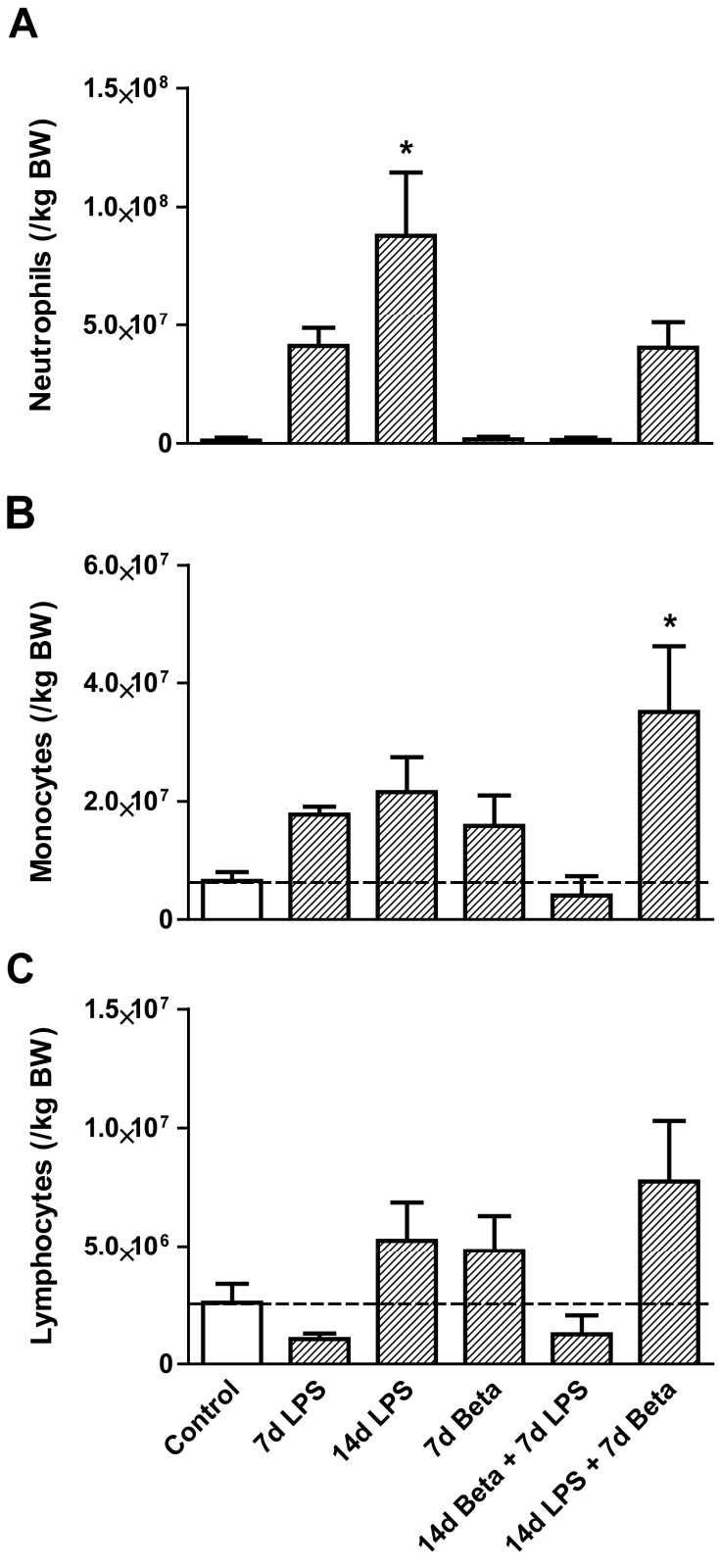

Figure 2: Differential cell count of the bronchoalveolar lavage. A: Neutrophil levels increased 14 days after the exposure to LPS. B: Combined exposure to LPS for 14 days and betamethasone for 7 days increased monocytes in the bronchoalveolar lavage. C: The lymphocyte count did not differ in any of the treatment groups compared to controls. * $p<0.05$ versus controls using a one-way ANOVA with Tukey`s post hoc test.

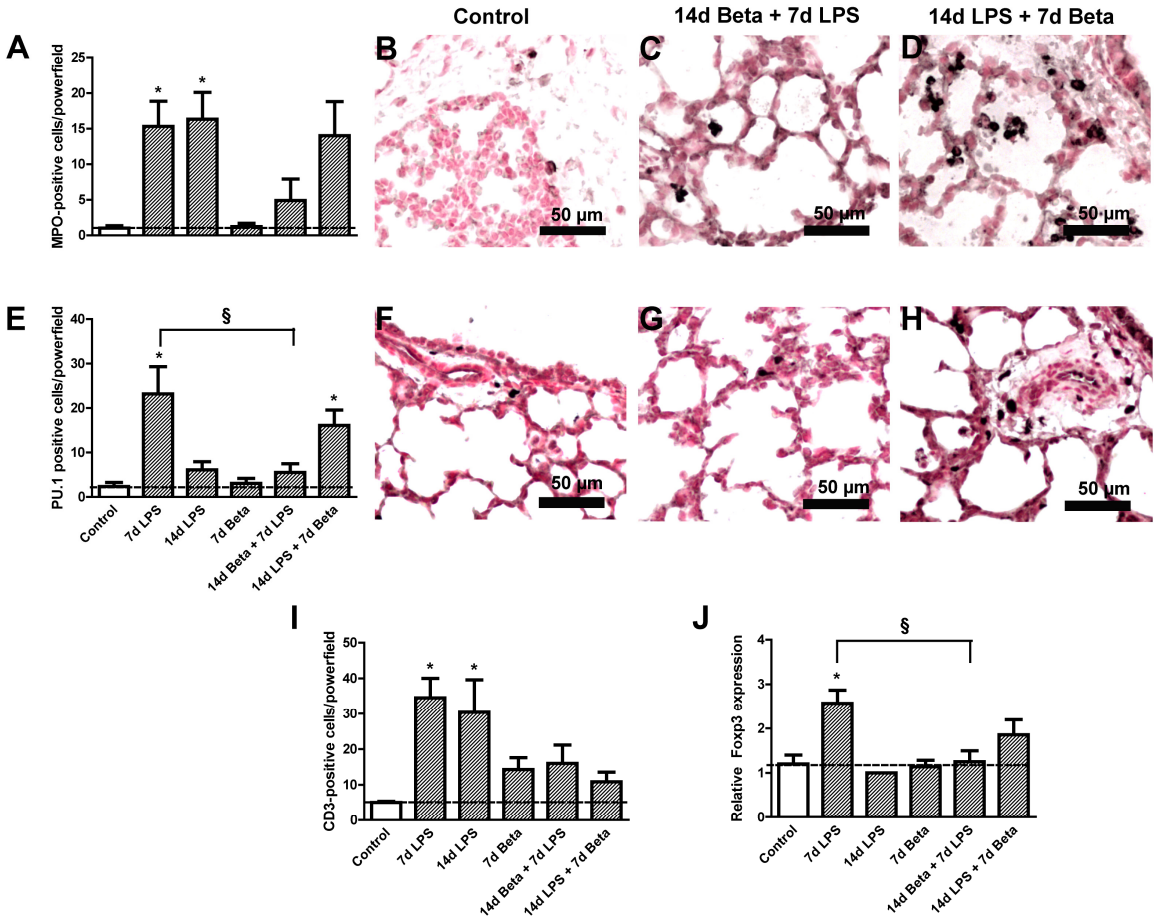

Figure 3: Characterization of inflammatory cells in the fetal lung tissue. A: Quantitation of myeloperoxidase expressing cells in lung sections per microscopic field corrected for the tissue fraction (see methods for details). Representative photomicrographs of immunostaining against myeloperoxidase using lung sections from B: Controls C: 14d Bethamethasone + 7d LPS D: 14d LPS + 7d Betamethasone. Myeloperoxidase (MPO) -positive cells increased in the lung tissue 14 days after LPS exposure compared to controls. E: Quantitation of PU.1 expressing cells in lung sections per microscopic field. Representative photomicrographs of immunostaining against PU.1 using lung sections from F: Controls G: 14d Bethamethasone + 7d LPS H: 14d LPS + 7d Betamethasone. LPS exposure for 7 days and combined exposure to LPS for 14 days and betamethasone for 7 days increased the number of PU.1-positive cells in the fetal lung. Betamethasone treatment 7 days before the LPS exposure prevented this increase. I: The number of CD3-positive cells increased significantly 7 and 14 days after LPS exposure compared to controls. J: Foxp3 expression increased 7 days after LPS exposure compared to controls. Betamethasone treatment before the LPS exposure prevented this increase. * $p<0.05$ versus controls § $p<0.05$ between experimental groups using a one-way ANOVA with Tukey's post hoc test.

Table 3: Cytokine and acute phase reactant expression in the fetal lung

Treatment group	*IL-1β* mRNA (fold increase)	*IL-6* mRNA (fold increase)	*IL-8* mRNA (fold increase)	*MCP-1* mRNA (fold increase)	Serum Amyloid A3 mRNA (fold increase)
Control	1.0±0.2	1.0±0.3	1.0±0.2	1.0±0.2	1.0±0.7
7d LPS	3.5±0.5*	0.9±0.1	4.7±0.8	3.7±0.4	29±10*
14d LPS	3.1±0.6	1.1±0.2	4.2±1.5	3.8±0.9	12±8.0
7d Beta	1.2±0.3	1.0±0.3	1.0±0.4	1.3±0.3	1.2±0.5
14d Beta + 7d LPS	1.8±0.4	0.8±0.1	2.1±0.8	1.7±0.5	4.3±3.0
14d LPS + 7d Beta	3.2±1.5	1.6±0.8	4.3±2.3	5.0±3.2	31±26

Data expressed as mean ± SEM. * $p<0.05$ versus controls using a one-way ANOVA with Tukey's post hoc test. LPS- Lipopolysaccharide, Beta- Betamethasone.

The mRNA levels of *TLR2* (Figure 4A) and *TLR4* (Figure 4B) more than doubled after 7 days of LPS exposure, but returned to control levels 14 days after exposure. Pre-treatment with betamethasone 7 days prior decreased the LPS induced increase in *TLR4* but not *TLR2* mRNA. Levels of TLR9 mRNA did not change in experimental groups compared to controls (Figure 4C).

Surfactant components

Exposure to LPS alone modestly and variably increased *SP-A* and *SP-B* mRNA levels (non-significant) (Table 4). However, the combined exposure to LPS followed by betamethasone resulted in a more consistent and significant increase in *SP-A* and *SP-B* mRNAs. *SP-C* mRNA expression did not change with either the betamethasone or LPS exposure. *SP-D* mRNA expression increased 14 days after the exposure to LPS alone or with betamethasone 7 d after LPS (Figure 5A). Consistent with the mRNA data, combined exposure to 14d LPS followed by betamethasone 7 d exposure significantly increased SP-D protein in the BALF (Figure 5B). To better assess the changes in the alveolar type II cells, we immunostained fetal lung sections using antibodies against TTF-1 and the precursor form of SP-C (Pro-SP-C) (39). TTF-1, a transcription factor for alveolar type II cells (39), did not change in any of the treatment groups indicating no change in the alveolar type II cell numbers after exposure to either LPS or betamethasone (Table 4). However, compared to controls, the number of Pro-SP-C-positive cells increased significantly 7, and 14 days after the exposure to LPS, and after 14d LPS+ 7d Beta, indicating maturation of alveolar type-II cells (Figure 6A-E).

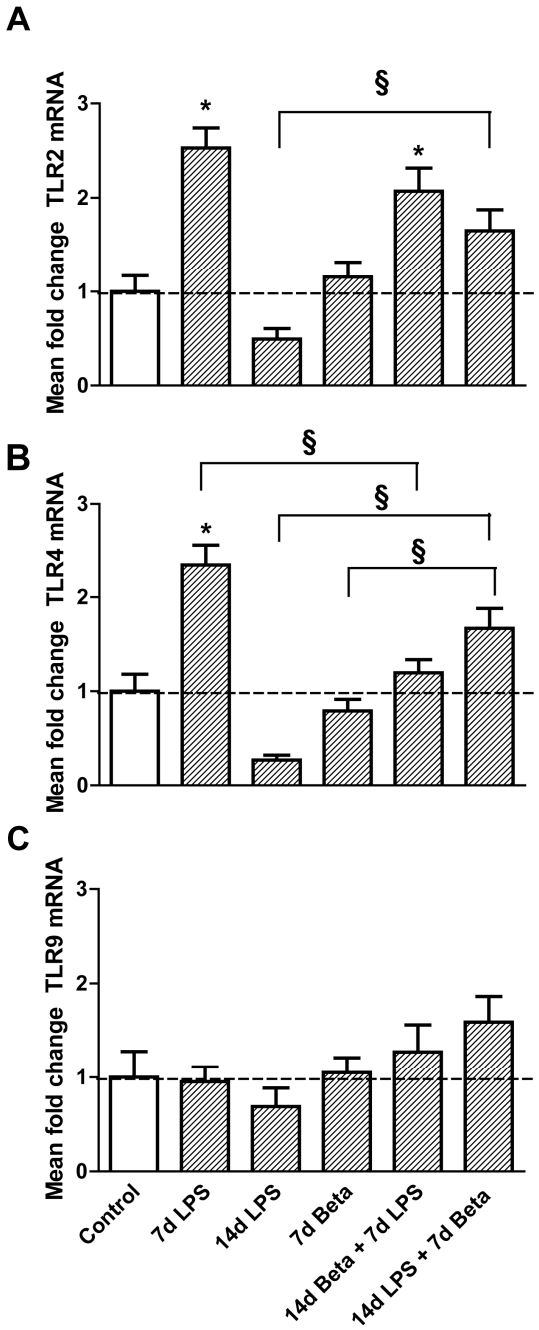

Figure 4: Expression of Toll-Like Receptors (TLR) 2, 4 and 9. *TLR2* (A) and *TLR4* (B) mRNA expression were up-regulated 7 days after LPS exposure, but returned to baseline 14 days after exposure to LPS. Betamethasone treatment before LPS exposure prevented increased *TLR4* expression, but not *TLR2* expression. *TLR2* and *TLR4* mRNA levels increased in the lungs of lambs which received betamethasone after LPS exposure, although these levels were not higher than levels measured in controls. C: *TLR9* was not differently expressed in experimental groups compared to controls. *p<0.05 versus controls; § p<0.05 between experimental groups using a one-way ANOVA with Tukey's post hoc test.

Table 4: Markers of lung maturation

Treatment group	SP-A mRNA (fold increase)	SP-B mRNA (fold increase)	SP-C mRNA (fold increase)	Pro-SP-C+ cells/HPF	TTF-1+ cells/HPF
Control	1.0±0.4	1.0±0.3	1.0±0.6	38±17	514±37
7d LPS	4.6±0.6	1.9±0.2	1.7±0.7	131±17*	501±25
14d LPS	7.5±2.6	2.4±0.4	0.4±0.1	83±11	307±14
7d Beta	1.4±0.2	1.1±0.1	0.2±0.1*	52±10	400±17
14d Beta + 7d LPS	5.6±3.3	1.8±0.6	1.0±0.3	67±11	269±19
14d LPS + 7d Beta	10.5±2.2*	2.7±0.3*	0.6±0.1	51±9	327±19

Data expressed as mean ± SEM. * p<0.05 versus controls using a one-way ANOVA with Tukey's post hoc test. LPS- Lipopolysaccharide, Beta- Betamethasone, HPF-High powerfield.

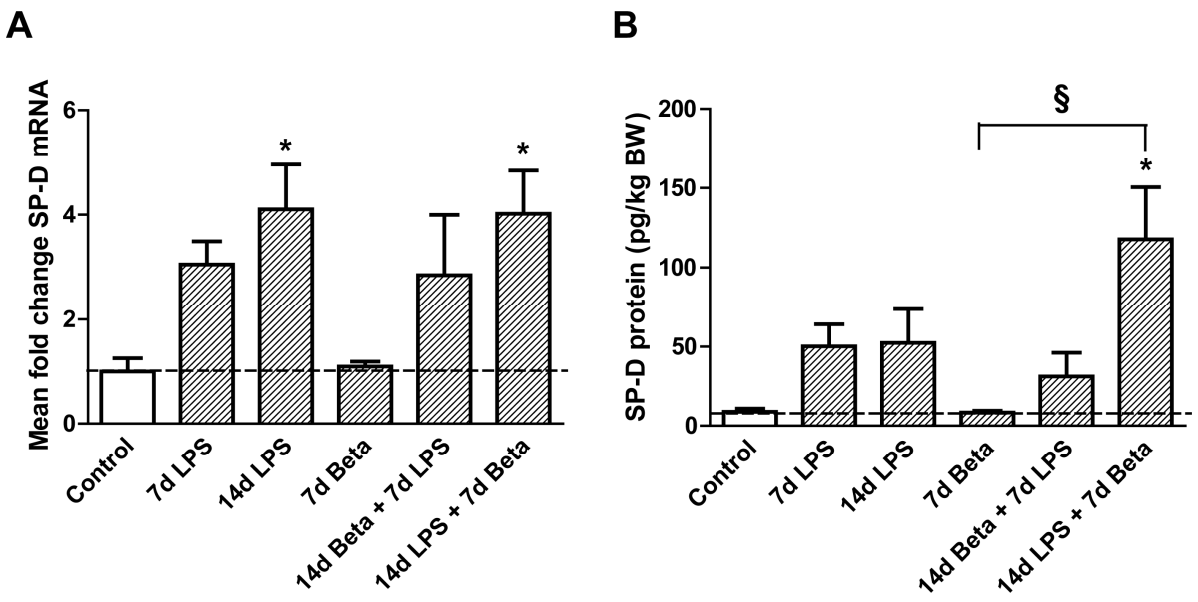

Figure 5: SP-D mRNA and protein expression. A: SP-D mRNA levels increased 14 days after the exposure to LPS irrespectively of the betamethasone post-treatment. B: Only combined exposure to LPS for 14 days and betamethasone for 7 days increased SP-D protein expression in the bronchoalveolar lavage fluid. Exposure to LPS for 14 days did not increase SP-D protein expression. * p<0.05 versus controls § p<0.05 between experimental groups using a one-way ANOVA with Tukey's post hoc test.

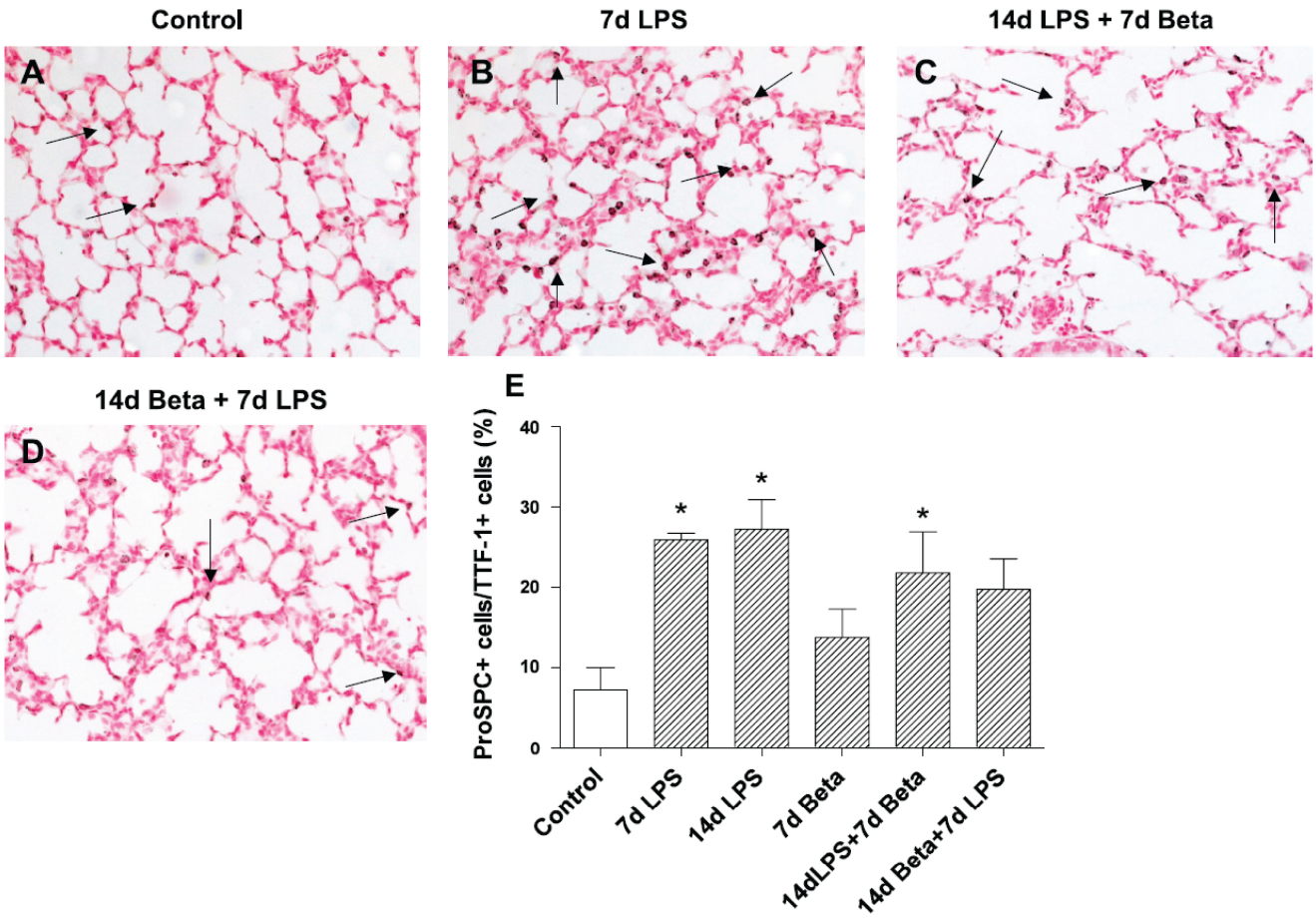

Figure 6: Pro-SP-C immunostaining in the lung. The number of pro-SP-C+ cells was expressed as a percentage of TTF-1 positive cells (data in table 4). Representative photomicrographs of immunostaining using lung sections from A: Controls B: 7d LPS C: 14d LPS + 7d Betamethasone D: 14d Bethamethasone + 7d LPS. E: Quantitation of Pro-SP-C expressing cells in lung sections per microscopic field using a 20X objective. LPS exposure increased Pro-SP-C expression in the alveolar type II cells in the lung. (*$p < 0.05$ vs. controls, scale bar is 50 µm).

Saturated phosphatidylcholine (Sat PC) is the major surfactant lipid (22). Exposure to intra-amniotic LPS for 7 or 14 days increased Sat PC in the fetal airspaces (Figure 7A). Betamethasone alone did not significantly increase Sat PC expression. However, the 14 days LPS exposure followed by betamethasone 7d had the highest airway Sat PC levels (Figure 7A). Consistent with increases in surfactant Sat PC, lung volumes, a measure of compliance, increased in all the LPS groups regardless of betamethasone exposure, with the highest lung volumes recorded in the 14d LPS followed by 7d betamethasone exposure group Figure 7B).

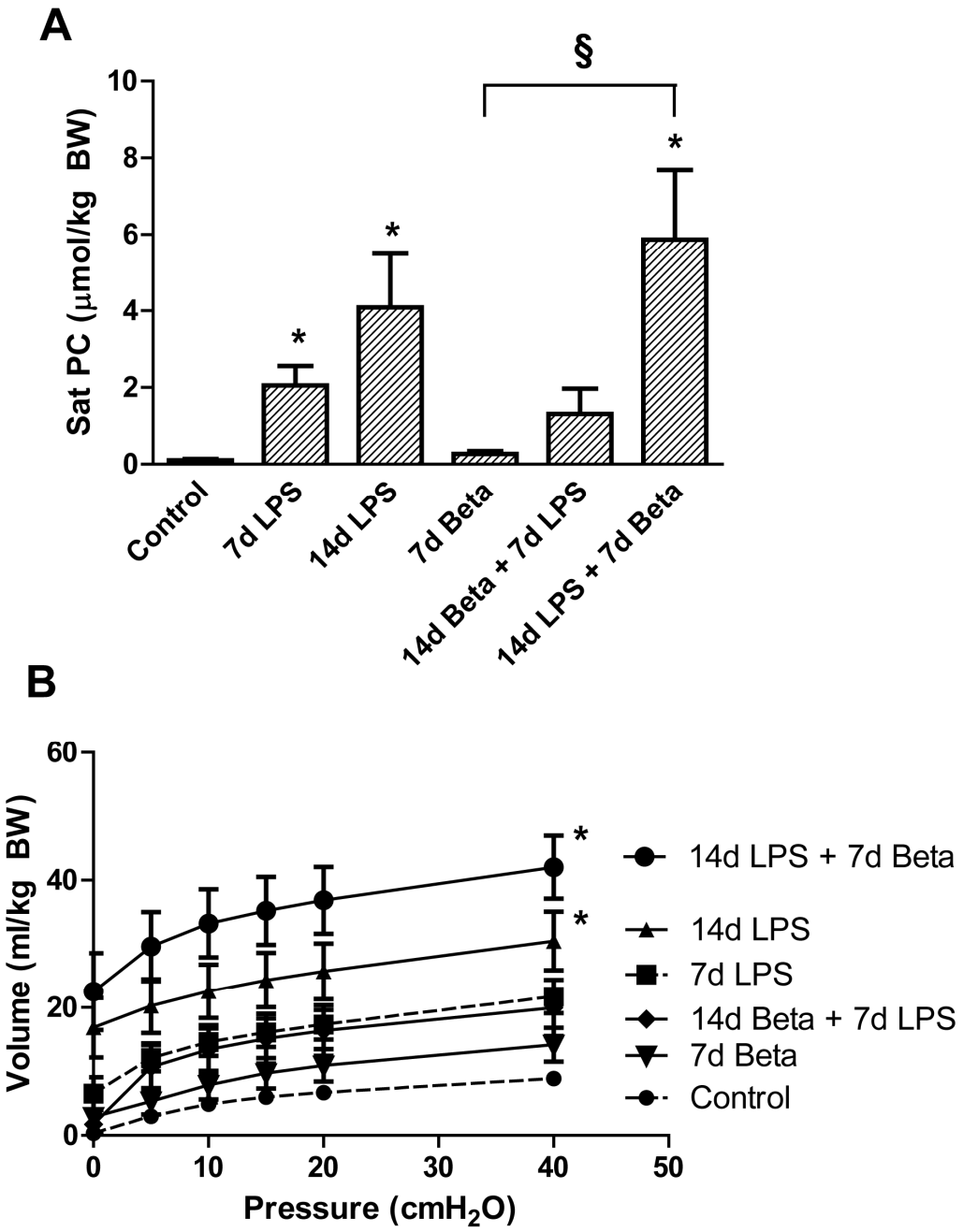

Figure 7: Saturated phosphatidylcholine (Sat PC) and pressure-volume curves. A: Sat PC levels in the bronchoalveolar lavage fluid were increased 7 and 14 days after exposure to intra-amniotic LPS. Betamethasone post-treatment 7 days after the exposure to LPS increased Sat PC levels further. B: The pressure-volume curve of animals after 14 days of LPS exposure was significantly higher compared to controls irrespectively of treatment with betamethasone. * $p<0.05$ versus controls; § $p<0.05$ between experimental groups using a one-way ANOVA with Tukey's post hoc test.

Discussion

Lung inflammation, increased airway surfactants and increased lung volumes consistent with clinical lung maturation are the major effects of experimental chorioamnionitis (33, 36). In this study we evaluated if the exposure to the commonly used anti-inflammatory drug

betamethasone altered the responses to LPS in the fetal lung. The major conclusion was that betamethasone when administered 7 days after intra-amniotic LPS did not inhibit lung inflammation but resulted in striking lung maturation. However, when betamethasone was given 7 days before intra-amniotic LPS, lung inflammation was suppressed and lung maturation was more modest.

Intra-amniotic LPS caused lung inflammation at 7 and 14 days after exposure. Both neutrophils and T-lymphocytes were recruited to the lung. Further, the neutrophils were activated as demonstrated by the MPO expression (10). The influx of pro-inflammatory cells was also accompanied by an increase in Foxp3-positive cells, a prototypic marker of the anti-inflammatory T-regulatory cells (13). The number of cells expressing PU.1 also increased which suggests maturation of lung monocytes, a characteristic for LPS-mediated pulmonary inflammation (35). Both the *TLR2* and *TLR4* mRNA levels increased in the lung tissue after LPS. Consistent with our previous results demonstrating an early rapid induction profile for cytokine expression (33), the mRNAs for pro-inflammatory cytokines were only modestly increased 7d after exposure in the present study. Interestingly, betamethasone treatment 7d prior to LPS significantly decreased LPS induced lung inflammation. However, when LPS exposure preceded betamethasone treatment, lung inflammation was not altered. We previously reported that a concomitant administration of maternal betamethasone and LPS resulted in an early suppression but later amplification of lung inflammation (28, 34). Thus, the timing of exposure to betamethasone in relation to the pro-inflammatory stimulus is a major determinant of modulation of lung inflammation.

Maternal betamethasone and intra-amniotic LPS have different pharmaco-kinetic profiles in the fetus. In a human study, peak fetal serum betamethasone levels were measured 1-2h after maternal betamethasone treatment with a return to baseline within 2 days of treatment (5). In fetal sheep, maternal betamethasone treatment resulted in peak fetal betamethasone levels 3 h after treatment with a decrease to 50 % of the peak levels at 6 h (9). In contrast, the half-life of endotoxin was measured as approximately 30 h in the amniotic fluid of preterm lambs after an intra-amniotic administration and exhibited first order kinetics of elimination (40). Neither antenatal betamethasone nor intra-amniotic LPS significantly change the levels of endogenous cortisol in the preterm fetus (21, 25). These results suggest that fetal betamethasone levels 7 d after maternal treatment must be negligible. Therefore, the inhibition of LPS induced fetal lung inflammation 7 d after maternal betamethasone suggests priming or conditioning of the inflammatory response cells rather than a straight forward drug-drug interaction. This interpretation is also consistent with our previous observation of an early suppression with a late amplification of lung inflammation with concomitant administration of maternal betamethasone and intra-amniotic LPS (28, 34). Our results also demonstrate that modulation of LPS induced fetal inflammation does not occur when betamethasone is administered after LPS.

Intra-amniotic exposure to LPS followed by betamethasone 7 days before delivery strikingly increased lung maturation as seen by increased Sat PC, SP-D and the increase in the pressure-volume curve. In contrast, betamethasone pre-treatment followed by exposure to LPS 7 days before delivery induced less lung maturation. The large effect of LPS on SP-D expression is consistent with recently reported up regulation of LPS-induced SP-D expression in fetal mice (44). SP-D is an innate host-defense molecule protecting the host against a number of pulmonary pathogens (12). Alveolar type II cell maturation was demonstrated by increased Pro-SPC expression. The expression of surfactant protein mRNAs for *SP-B* increased but *SP-C* was unchanged after LPS. Betamethasone alone did not increase expression of any of the surfactant protein mRNAs. We previously reported a differential effect of LPS on *SP-B* and *SP-C* mRNA levels (3). In vitro, these two mRNAs are differentially regulated by betamethasone (4). The more pronounced effect of LPS compared to betamethasone in increasing mRNA levels of surfactant proteins in this study is consistent with previous reports showing a rapid but reversible effect of betamethasone, but a more lasting effect of intra-amniotic LPS on surfactant protein mRNA expression (3, 45). Previous reports suggest that the effects of simultaneous exposure to explants of fetal rabbits to IL-1α and glucocorticoids could be stimulate or inhibit surfactant protein mRNA depending on the gestational age (46). Taken together, the combined effects of a pro-inflammatory exposure and glucocorticoids on the fetal lung are variable and context dependent including gestational age and the order of exposure.

We reported previously that intra-amniotic LPS but not maternal betamethasone improved lung compliance, ventilatory efficiency index and the alveolar wash saturated phosphatidyl choline pool sizes 15 d after treatment (3, 22). We did not study the effects of betamethasone alone 14 d after exposure, because of high rate of abortions in this group. Interestingly, the highest expression of surfactant components and the consequent increase in lung volumes was in the group exposed to LPS for 14 d along with betamethasone 7 d before delivery. This group of animals also had the most pronounced lung inflammation. These results are consistent with our previous studies demonstrating that lung inflammation was required for LPS induced lung maturation (30, 31).

Clinical studies indicate that maternal glucocorticoids can decrease fetal weight and head size (14). We reported previously that simultaneous exposures of fetal sheep to maternal corticosteroid and intra-amniotic LPS protected the fetus from the growth restriction caused by the antenatal corticosteroids (41). In this experiment, the maternal corticosteroid exposure did not decrease fetal weight, a result that probably represents animal variability. The new observation is that the combination of 14 day LPS + 7 day Beta caused fetal growth restriction. Since exposure to intrauterine inflammation is the major cause for preterm labor and cervical dilation leading to preterm birth, pretreatment with betamethasone will rarely be an option to diminish or counteract inflammation in the clinical setting. An inference from the study is that prenatal betamethasone in conjunction with inflammation promotes the maturity of the surfactant system resulting in reduced RDS, but does not prevent BPD as

noted in clinical studies (8, 48). However, the interactions of LPS and Beta on fetal growth are complex and not fully understood, and this observation needs verification by replication.

Antenatal corticosteroids are routinely administered to mothers who are at risk of preterm birth to mature the fetal organs, suppress inflammation and thus reduce perinatal morbidity and mortality (1). Antenatal steroids also reduce adverse neonatal outcome after preterm birth associated with chorioamnionitis (15). Our experiments do not identify new concerns for the use of antenatal corticosteroids. It remains unknown if these antenatal corticosteroids also prevent or amplify the developmental and structural complications in the fetal lung, which are induced by infection/inflammation (11, 49). In summary, this study provides further molecular evidence into the protective effects of antenatal corticosteroids on the fetal lung and its interactions with pro-inflammatory stimuli in the setting of chorioamnionitis.

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CHAPTER 3

LPS-induced chorioamnionitis and antenatal corticosteroids modulate Shh signaling in the ovine fetal lung

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Abstract

Rationale Chorioamnionitis and antenatal corticosteroids mature the fetal lung functionally but disrupt late gestation lung development. Because Sonic Hedgehog (Shh) signaling is a major pathway directing lung development, we hypothesized that chorioamnionitis and antenatal corticosteroids modulated Shh signaling resulting in an altered fetal lung structure.

Methods Time-mated ewes with singleton ovine fetuses received an intra-amniotic injection of lipopolysaccharide (LPS) and/or maternal intra-muscular betamethasone 7 and/or 14 days before delivery at 120 days gestational age (GA) (term=150 days GA).

Results Intra-amniotic LPS exposure decreased *Shh* mRNA levels and Gli1 protein expression which was counteracted by both betamethasone pre- or post-treatment. mRNA and protein levels of fibroblast growth factor 10 and bone morphogenetic protein 4, which are important mediators of lung development, increased 2-fold and 3.5-fold respectively, 14 days after LPS exposure. Both 7 day and 14 day exposure to LPS changed the mRNA levels of elastin gene *ELN* and collagen type I genes *Col1A1* and *Col1A2* which resulted in fewer elastin foci and increased collagen type I deposition in the alveolar septa. Corticosteroid post-treatment prevented the decrease in *ELN* mRNA and increased elastin foci and decreased collagen type I deposition in the fetal lung.

Conclusion Fetal lung exposure to LPS was accompanied by changes in key modulators of lung development resulting in abnormal lung structure. Betamethasone treatment partially prevented the changes in developmental processes and lung structure. This study provides new insights into clinically relevant prenatal exposures and fetal lung development.

Introduction

Bronchopulmonary dysplasia (BPD), a disease of impaired lung development, is the most common adverse lung outcome of preterm birth (2, 30). BPD is associated with fetal lung inflammation which can be initiated by chorioamnionitis, an intra-uterine bacterial infection of the placental membranes and amniotic fluid that is often clinically silent (21). Chorioamnionitis can induce a potentially harmful inflammatory response in the immature fetal lungs which disrupts lung septation and vascular development leading to a decreased lung surface area (16).

Antenatal corticosteroids are given to mothers at risk of imminent preterm birth to induce lung maturation in the fetus which increases neonatal survival, but do not decrease BPD (4, 14). Because the incidence of chorioamnionitis is about 60% for very preterm babies, the administration of maternal antenatal corticosteroids in the presence of chorioamnionitis is common and standard of care (3). Although antenatal corticosteroids cause functional lung maturation, they also can inhibit lung development (54). As a result, a large number of premature infants are exposed *in utero* to both pro- and anti-inflammatory stimuli which each alter normal fetal lung development and might predispose the infants to the development of BPD (10). The molecular mechanisms by which chorioamnionitis and antenatal corticosteroids influence these lung developmental processes are largely unknown.

Sonic Hedgehog (Shh) signaling is critical for lung development as Shh-null mice have hypoplastic lungs and die due to respiratory failure (32). During lung development, Shh expression is localized to the epithelium and activates Gli transcriptional activators Gli1, Gli2 and Gli3 (5). The Shh pathway regulates the expression of lung growth factors such as fibroblast growth factor 10 (FGF10) and bone morphogenetic protein 4 (BMP4) which both mediate branching and myofibroblast differentiation (52).

We hypothesized that chorioamnionitis and/or antenatal corticosteroids modulate Shh signaling to alter fetal lung structural development. We evaluated this signaling pathway after LPS-induced chorioamnionitis in a 120 days gestational age (GA) preterm lamb model during a stage of early alveolar septation. Fetal sheep were exposed *in utero* to intra-amniotic lipopolysaccharide (LPS) from gram negative bacteria and/or antenatal betamethasone, a corticosteroid used clinically to induce lung maturation (28, 41). We correlated Shh signaling components with markers for lung damage (heat shock protein (HSP)70, cell proliferation (Ki67) and changes in the lung structural proteins elastin and collagen, which are crucial for alveolar septation (9, 25, 49).

Materials and methods

Animal model and sampling protocol

All studies were approved by the Animal Ethics Committees at The University of Western Australia and Cincinnati Children`s Hospital Medical Center (animal ethics protocol RA/3/100/830). The experimental design of this study was published previously (29). Time-mated Merino ewes with singleton fetuses were randomly assigned to one of six treatment groups to receive an intra-amniotic (IA) injection of lipopolysaccharide (LPS) (10 mg *Escherichia Coli* 055:B5, Sigma Chemical, St. Louis, MO, USA) and/or an intra-muscular injection of betamethasone (Beta) (Celestone Soluspan, Schering-Plough, North Ryde, New South Wales (NSW), Australia, 0.5 mg/kg maternal weight) and/or an equivalent injection of saline for control animals at 107 days and/or 114 days GA. All ewes in this study received a single intra-muscular injection of 150 mg medroxyprogesterone acetate (Depo-Provera, Kenral, NSW, Australia) at 100 days GA to prevent preterm birth induced by betamethasone treatment. Lambs were surgically delivered at 120 days GA (term = 150 days GA) and euthanized after birth. Lung tissue from the right lower lobe (RLL) was snap frozen and the right upper lobe (RUL) was inflation-fixed in 10% buffered formalin for 24 hours.

RNA extraction and real-time PCR

Total RNA was extracted from frozen lung tissue of the RLL using the SV Total RNA Isolation system (Z3100, Promega, Madison, USA) according to the manufacturer's instructions. Genomic DNA contamination was removed by treatment with RQ1 DNase (M610A, Promega) and the RNA was tested for the presence of genomic *GAPDH*, a housekeeping gene. Briefly, PCR amplification for the detection of genomic DNA was performed with DNA Taq Polymerase (M124B, Promega) at 95°C for 5 minutes followed by 40 cycles at 95°C for 30 seconds, 55°C for 45 seconds and 72°C for 30 seconds. Total RNA was used as a template. PCR products were analyzed on a 1.5% agarose gel. Total RNA was reverse transcribed with the First Strand cDNA synthesis kit (4379012001, Roche-Applied, Mannheim, Germany) according to manufacturer's instructions using anchored oligo-primers. Primers for real-time PCR (RT-PCR) were constructed based on published ovine or bovine cDNA sequences (Table 1). Dilution experiments were performed to ensure similar PCR amplification efficiency of the primers. RT-PCR reactions were performed in duplicate with the LightCycler 480 SYBR Green I Master mix (4707516001, Roche-Applied) on a LightCycler 480 Instrument according to the manufacturer's instructions. RT-PCR results were normalized to cyclophilin A, a housekeeping gene, and mean fold changes in mRNA expression were calculated by the ΔΔCt-method (33).

Table 1: Primers used for RT-PCR

Gene		Sequence (5`-3`)	Amplicon size (bp)	T^m	Accession code (RefSeq)
Col1A1	Fw	GAAGAAGACATCCCACCAG	125	60°C	NM_001034039.1
	Rv	GTCCTTAAGTTCGTCGCAG			
Col1A2	Fw	GGCTCAACCTGAAGACATCC	150	59°C	EF114225.1
	Rv	TCTCCTACCCAGACATGCTTC			
ELN	Fw	CCAAATTCGGTGCTGCTG	144	60°C	NM_175772.1
	Rv	ACTCCAACACCTGGGACTC			
Shh	Fw	ACTGGAGCGGACCGGCTGAT	82	68°C	XM_614193.3
	Rv	CCGGCCACTGGCTCATCAC			
Gli1	Fw	AATCTGAAGACGCACCTG	137	60°C	NM_001099000.1
	Rv	GTAGGGCTTCTCATTGGA			
Gli2	Fw	CCTGGAGAACCTGAAGAC	147	60°C	NM_001192250.1
	Rv	GATGTAGGGTTTCTCGTTGG			
Gli3	Fw	AGAAGCCTCACAAATGCAC	197	60°C	XM_002686896.1
	Rv	ACACATATGGTTTCTCGTTGG			
FGF10	Fw	TGCCCGTACAGTATCCTG	220	60°C	NM_001009230.1
	Rv	GCCACATACATTTGCCTC			
BMP4	Fw	ACCACGAAGAACATCTGGAG	173	61°C	NM_001110277.1
	Rv	TTATACGATGAAAGCCCTGC			
Cyclophilin A	Fw	TTATAAAGGTTCCTGCTTTCACAGAA	93	60°C	NM_178320.2
	Rv	ATGGACTTGCCACCAGTACCA			

Protein extraction and enzyme-linked immunosorbent assay (ELISA) of HSP70

Frozen RLL lung tissue was homogenized (PRO Quick Connect Generators part no. 02-07095; PRO Scientific Inc., Oxford, CT) in ice-cold RIPA buffer (R0278, Sigma Aldrich) containing 0.1% protease inhibitors (p9599, Sigma Aldrich) and subsequently centrifuged at 12x RCF for 5 minutes at 4°C (31). HSP70 was measured with an R&D DuoSet ELISA development kit (human/mouse/rat total HSP70: DYC1663, R&D Systems, Minneapolis, MN, USA) according to manufacturer`s instructions. HSP70 protein concentrations were calculated per kilogram bodyweight.

Immunohistochemistry

Paraffin embedded RUL lung sections (4 µm, transverse) were stained for Ki67 (M7240, DAKO, Denmark), Gli1 (ab49314, Abcam, Cambridge, UK) and BMP4 (sc-6896, Santa Cruz Biotechnology). Briefly, the sections were deparaffinized in an ethanol series and endogenous peroxidase-activity was blocked by incubation with 0.5% H_2O_2 in 1x phosphate buffered saline (PBS, pH 7.4). Antigen retrieval was performed by incubating the sections in heated citrate buffer (10 mM, pH 6.0) for 30 minutes. In order to block aspecific binding, the slides were incubated with 20% normal goat serum (NGS) in PBS. Sections were incubated overnight at 4°C with the diluted primary antibody (Ki67 1:50, Gli1 1:500, BMP4 1:500). After

incubation with the appropriate secondary antibody, immunostaining was enhanced with Vectastain ABC peroxidase Elite kit (PK-6200, Vector Laboratories, Burlingame, USA) and stained with nickel sulfate-diaminobenzidine (NiDAB). Subsequently, the sections were rinsed in Tris/saline and incubated with Tris/cobalt. After counterstaining with 0.1% Nuclear Fast Red, the sections were washed, dehydrated and coverslipped. All slides were stained at the same time under the same conditions.

Evaluation was performed by light microscopy (Axioskop 40, Zeiss, Germany) with LeicaQWin Pro v.3.4.0 software (Leica Microsystems, Germany). Alveolar Ki67 and Gli1 staining was scored by blinded observers with a semi-quantitative scoring system: 1, little staining; 2, some staining; and 3, heavy staining. BMP4 staining was semi-quantitatively scored in three representative bronchioli using Image J software (Rasband, W.S., Image J US National Institutes of Health, Bethesda, Maryland, USA) and represented as a percentage of the entire bronchiole surface area.

Elastin and collagen staining

Elastin and collagen stainings were performed each on 4 paraffin sections of the RUL per animal (4 µm, transverse). For the visualization of elastin, the sections were deparaffinized in an ethanol series and incubated in Hart`s staining solution (70% ethanol, 10% Weigerts Resorcine-Fuchsine (2E 030, Chroma, Münster, Germany) and 2% hydrochloric acid) overnight at room temperature. After rinsing with water, the sections were incubated in 0.25% acetic acid for 3 minutes at room temperature. Subsequently the sections were washed and dehydrated. For the detection of collagen fibers, the sections were deparaffinized and incubated in 0.2% phosphomolybdic acid for 5 minutes. Sections were placed in a Sirius Red solution for 90 minutes in the dark. After rinsing with 0.01 M HCl for 3 minutes, the sections were washed, dehydrated and coverslipped. Evaluation was performed by light microscopy (Zeiss, Axioskop 40) with LeicaQWin Pro v.3.4.0 software. The number of elastin foci and the percentage of collagen fibers in the total lung surface area were quantified using 4 paraffin sections per animal, 12 representative images per section across septa at 200x magnification by a blinded observer using specialized LeicaQWin Pro v.3.4.0 software.

Data analysis

Results are given as means ± standard error of mean (SEM). The groups were compared using one-way ANOVA with Dunnett's or Tukey's test for post-hoc analysis as appropriate. Statistical analysis was performed by GraphPad Prism v5.0. Significance was accepted at $p<0.05$.

Results

Lung damage and cell proliferation

Characteristics of the animals and the pulmonary inflammatory and maturation response to LPS-induced chorioamnionitis and/or antenatal corticosteroids were reported previously (29). Lung injury due to the exposure to LPS was assessed by measurement of heat shock protein 70 (HSP70) in the lung tissue. HSP70 protein expression was not increased in any of the experimental groups compared to control (Figure 1A). To assess cell proliferation, lung tissue was stained for Ki67, a marker of mitotic cells. There were increased proliferating cells, which by morphologic evaluation could be discerned as immune cells, 7 and 14 days after the exposure to LPS (Figure 1B). Representative images are shown for controls (Figure 1C) and 7 day LPS exposed lungs (Figure 1D).

Changes in Shh signaling after intra-uterine LPS exposure

Shh mRNA levels decreased to less than 25% of control value after 7 and 14 days of LPS exposure (Figure 2E). Betamethasone pre- or post-treatment prevented the decrease in *Shh* mRNA. In addition, we analyzed the expression of Gli1 and Gli2, which are components of the Shh pathway. *Gli1* mRNA expression had a similar decreased expression at 7 and 14 days following LPS exposure (Figure 2F). Gli1 protein expression was mainly detected in the bronchiolar and alveolar epithelium in controls (Figure 2A). Exposure to LPS for 7 or 14 days selectively decreased Gli1 protein expression in the alveolar epithelium (Figure 2B). Betamethasone pre- or post-treatment again prevented this decline (Figure 2G). Representative images are shown for controls (Figure 2A), 14 day LPS exposed lungs (Figure 2B), 7 day betamethasone exposed lungs (Figure 2C) and 14 day LPS and 7 day betamethasone exposed lungs (Figure 2D). *Gli2* mRNA expression had similar trends towards declines after LPS exposure (Figure 2H).

Levels of FGF10 and BMP4, which are two important Shh regulated mediators of lung development, were also assessed. Both *FGF10* and *BMP4* mRNA increased 14 days after LPS exposure, by 2-fold and 3.5 fold respectively (Figure 3A and 3B). Exposure to betamethasone after LPS exposure lowered *FGF10* and *BMP4* mRNA. BMP4 protein expression was mainly localized in the bronchial epithelial cells, which corresponds with recent data obtained in adult lung tissue (35, 42). Immunohistochemical analysis of BMP4 expression in bronchioli revealed that BMP4 was decreased 7 days after LPS exposure, and showed a trend towards increased expression at 14 days after LPS exposure (Figure 3C). Treatment with betamethasone before LPS exposure prevented the decrease in BMP4 levels seen after 7 day LPS exposure only. Treatment with betamethasone 7 days after the LPS exposure decreased BMP4 levels.

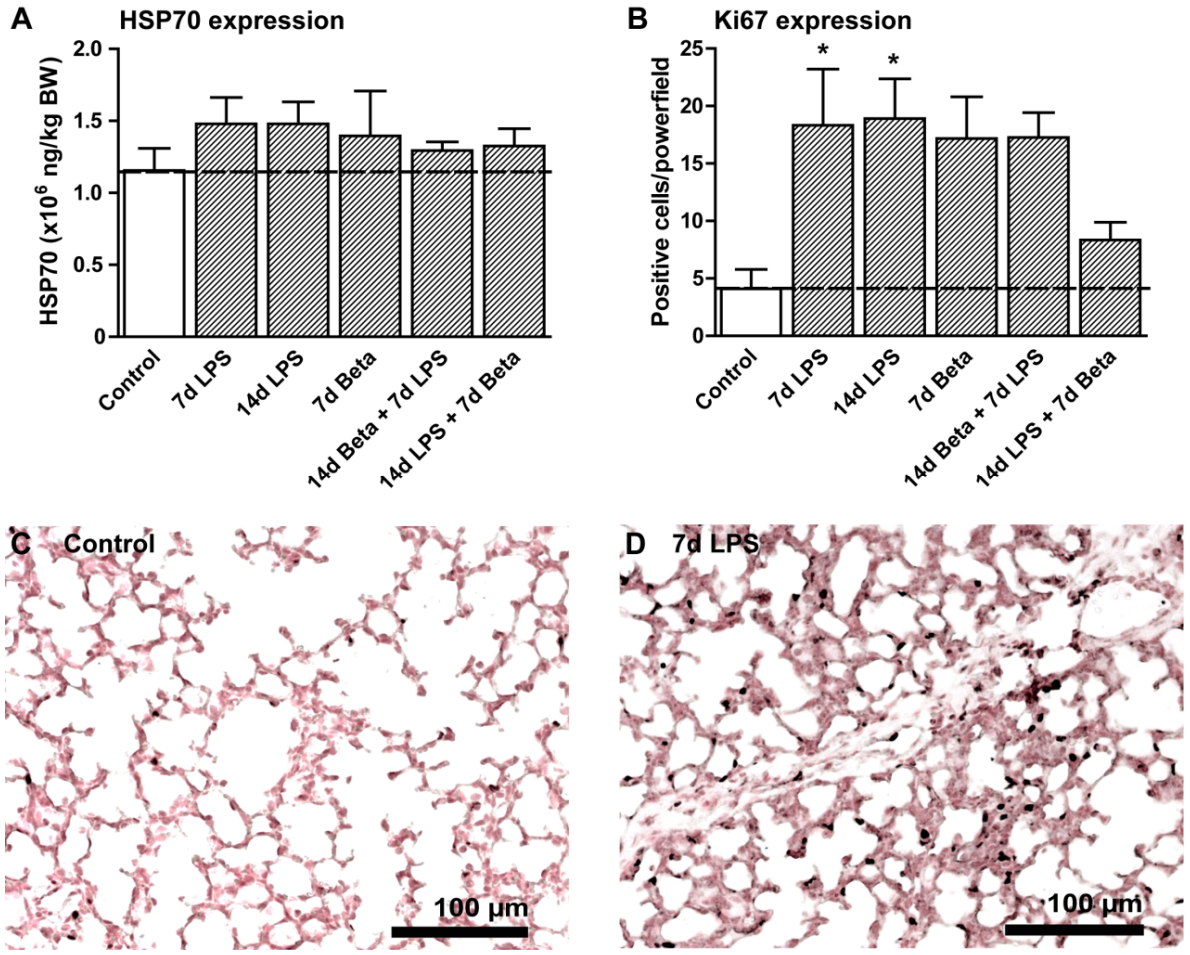

Figure 1: Lung injury and cell proliferation. **A:** Protein levels of Heat Shock Protein (HSP)70 did not change in homogenates of LPS and/or betamethasone (Beta) exposed fetal lungs. **B:** The number of Ki67-positive cells in the alveoli increased after LPS exposure. Pre- and particularly post-treatment with Beta partially prevented this increase. Alveolar Ki67 expression in controls (**C**) and 7d LPS exposed animals (**D**). BW – bodyweight. * $p<0.05$ versus controls using a one-way ANOVA with Tukey's post hoc test.

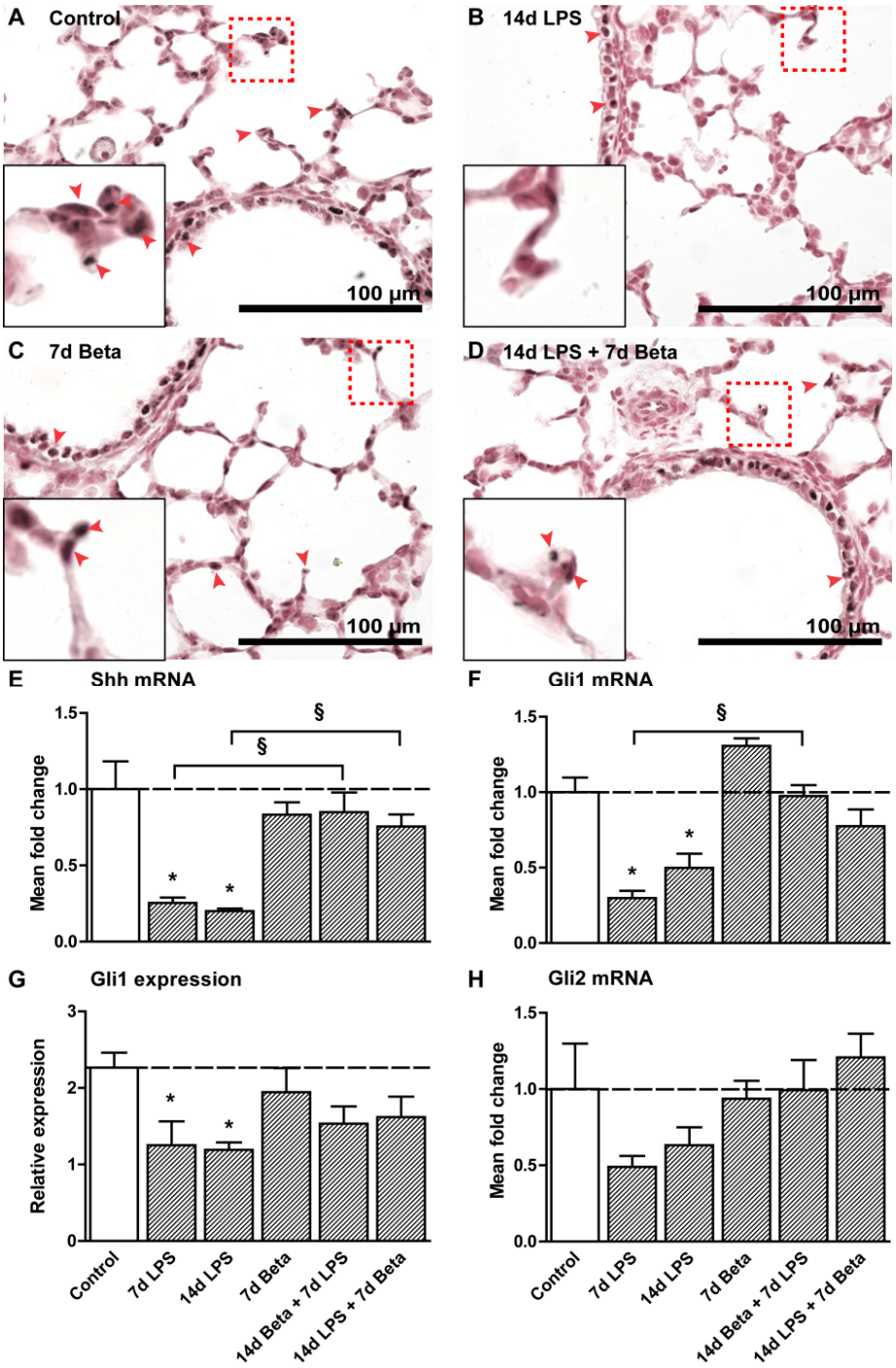

Figure 2: Inhibition of the Sonic Hedgehog (Shh) pathway. Gli1 expression in alveolar and bronchial tissue as seen in controls (**A**), 14 days after LPS exposure (**B**), 7 days after betamethasone (Beta) treatment (**C**) and after a combination of 14 days LPS followed by post-treatment with Beta (**D**). **E:** Expression of *Shh* was decreased after 7 and 14 days LPS exposure. Both pre- and post-treatment with betamethasone (Beta) normalized *Shh* mRNA levels compared to controls. **F:** *Gli1* mRNA levels were decreased in LPS exposed lungs. Both pre- and post-treatment with Beta normalized *Gli1* mRNA levels compared to controls. **G:** Gli1 protein expression as scored in the alveoli in lung sections decreased after 7 and 14 day LPS exposure. Pre- or post-treatment could partially attenuate this decrease. **H:** Levels of *Gli2* mRNA in experimental groups did not differ significantly from controls. * $p<0.05$ versus controls and § $p<0.05$ between experimental groups using a one-way ANOVA with Tukey's post hoc test.

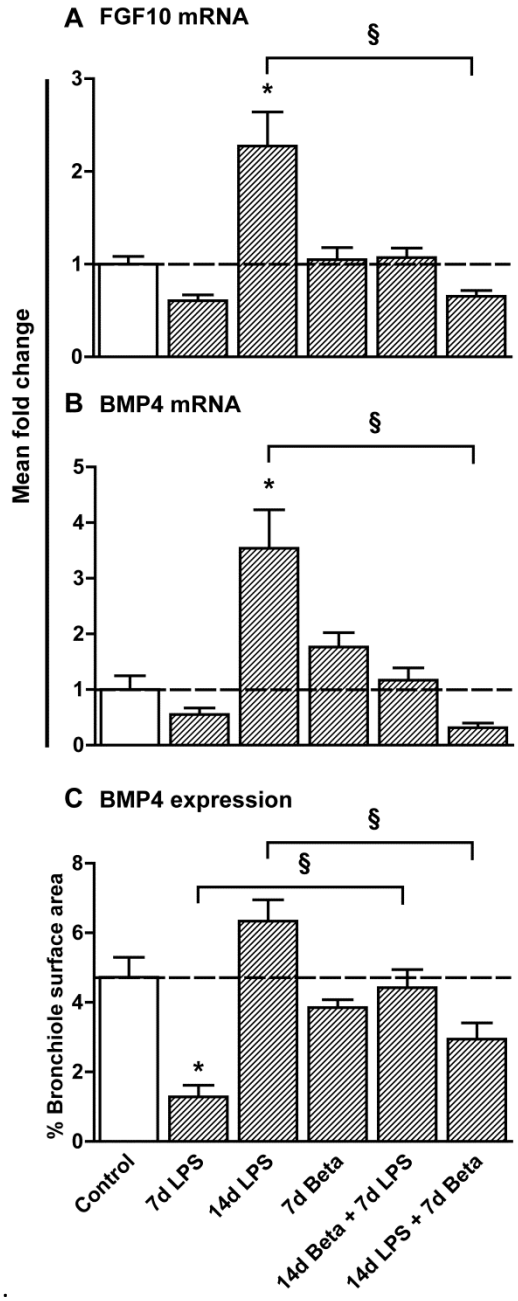

Figure 3: Expression of Fibroblast Growth Factor (FGF)10 and Bone Morphogenetic Protein (BMP)4. **A:** mRNA levels of *FGF10* were increased two-fold 14 days after LPS exposure. Post-treatment with betamethasone (Beta) normalized *FGF10* levels compared to controls. **B:** mRNA levels of *BMP4* were increased 3.5-fold 14 days after LPS exposure. Post-treatment with Beta normalized *BMP4* levels compared to controls. **C:** Immunohistochemical analysis of BMP4 expression in bronchioli decreased after 7 days of LPS exposure, but a recovery of BMP4 14 days after LPS exposure. Pre-treatment with Beta before LPS exposure prevented a drop in BMP4 levels. * $p<0.05$ versus controls and § $p<0.05$ between experimental groups using a one-way ANOVA with Tukey's post hoc test.

Expression of lung structural proteins

Elastin foci were quantified in lung sections as an assessment of secondary septation. Representative images are shown for control (Figure 4A), 7d LPS (Figure 4B), 14d LPS (Figure

4C), 7d Beta (Figure 4D), 14d Beta + 7d LPS (Figure 4E) and 14d LPS + 7d Beta (Figure 4F) lambs. The number of elastin foci decreased in the lungs of LPS exposed groups lambs (Figure 4G). Pre-treatment with betamethasone minimized the decrease in elastin foci. Post-treatment with betamethasone after LPS exposure increased elastin foci in the fetal lung. *ELN* mRNA first decreased by 50% 7 days after LPS exposure followed by a 50% increase 14 days after LPS exposure compared to controls (Figure 4H). Betamethasone pre-treatment followed by 7 day LPS exposure increased *ELN* mRNA by 50% compared to controls. No change in *ELN* mRNA was detected in the 14d LPS + 7d Beta animals.

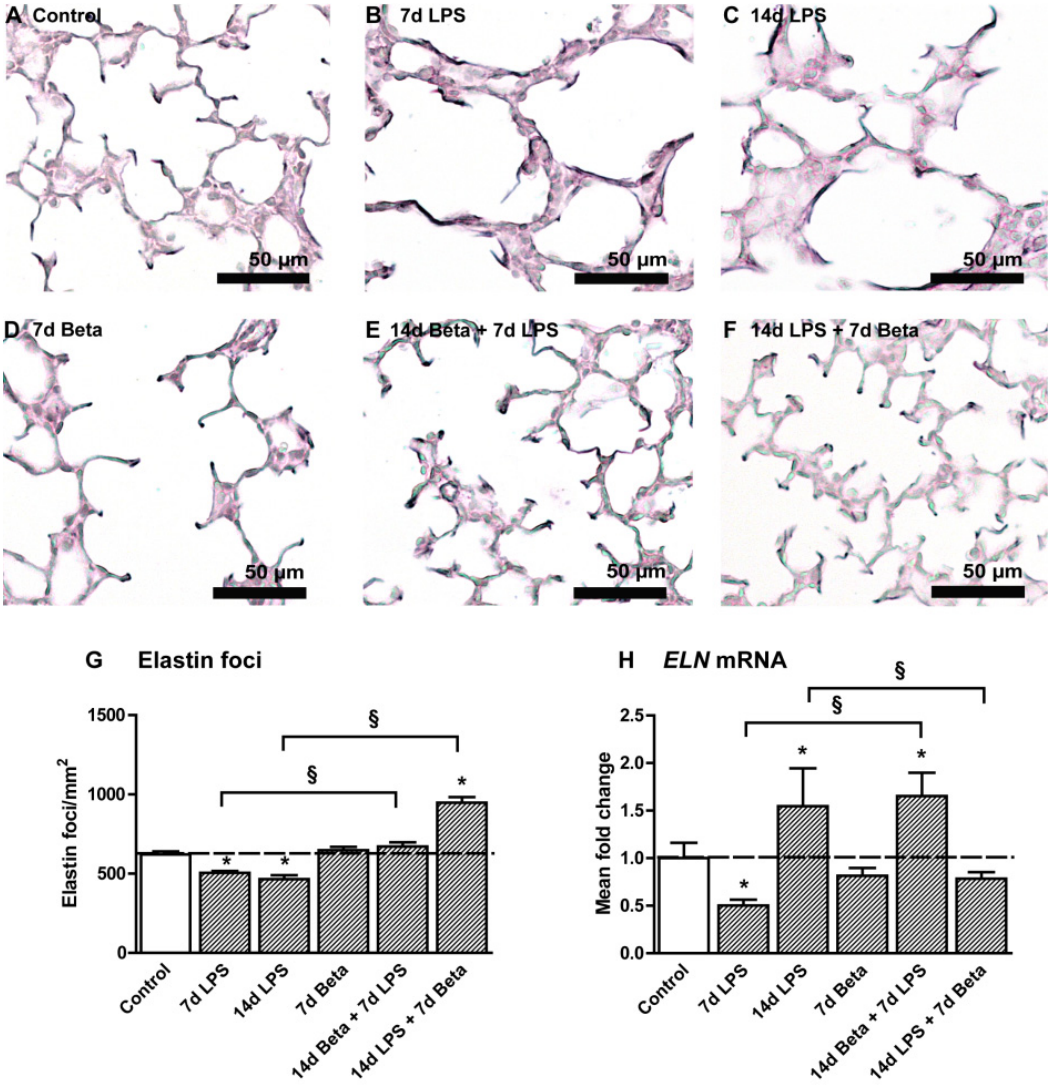

Figure 4: Altered expression of elastin. Elastin deposition in the alveoli of control (**A**), 7d LPS (**B**), 14d LPS (**C**), 7d Beta (**D**), 14d Beta + 7d LPS (**E**) and 14d LPS + 7d Beta (**F**) lambs. **G:** The number of elastin foci per mm^2 tissue was decreased in LPS exposed lambs. Pre-treatment with Beta could prevent a significant decrease in elastin foci. Post-treatment with Beta after LPS exposure increased the number of elastin foci. **H:** *ELN* mRNA levels decreased by 50% in 7d LPS lambs but increased in 14d LPS exposed lambs. Pre-treatment with Beta before LPS exposure resulted in a 60% increase in *ELN* mRNA levels. Post-treatment with Beta prevented the increase in *ELN* mRNA. * $p<0.05$ versus controls and § $p<0.05$ between experimental groups using a one-way ANOVA with Dunnett's post hoc test.

Representative images of the collagen deposition in the fetal lungs are shown for controls (Figure 5A), 7d LPS (Figure 5B), 14d LPS (Figure 5C), 7d Beta (Figure 5D), 14d Beta + 7d LPS (Figure 5E) and 14d LPS + 7d Beta (Figure 5F) lambs. *Col1A1* mRNA increased more than 2-fold after combined 14 day Beta and 7 day LPS exposure (Figure 5H). Collagen type I deposition increased after 14 days of LPS exposure (Figure 5G). In contrast, 14 day LPS exposure followed by betamethasone post-treatment resulted in a significant decrease of collagen type I deposition. Betamethasone pre-treatment followed by 7 day LPS exposure increased collagen type I deposition in the fetal lung. In contrast, 14 day LPS exposure followed by betamethasone post-treatment significantly decreased mRNA levels of *Col1A1* similar to the collagen deposition in the lung. mRNA levels of collagen type I gene *Col1A2* decreased to 50% after 14 days of LPS exposure irrespective of betamethasone post-treatment (Figure 5I). Interestingly, 7 day betamethasone exposure only also decreased *Col1A2* mRNA by around 40%. Further computerized morphometric analyses are reported elsewhere and did not show differences (29).

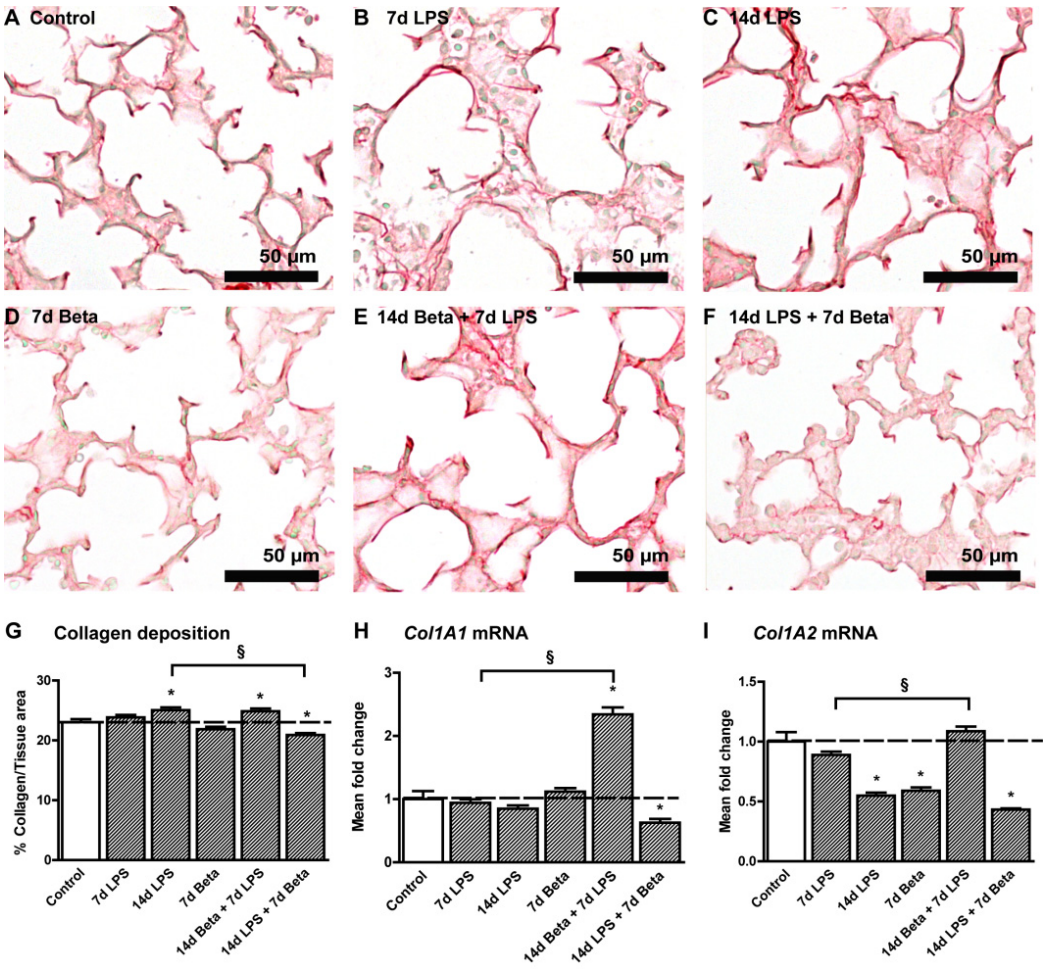

Figure 5: Collagen I expression. Collagen I deposition in the alveoli of control (**A**), 7d LPS (**B**), 14d LPS (**C**), 7d Beta (**D**), 14d Beta + 7d LPS (**E**) and 14d LPS + 7d Beta (**F**) lambs. **G:** 14 day LPS exposure and 14d Beta + 7d LPS exposure increased the percentage of collagen type I tissue in the fetal lung. Post-treatment with Beta after LPS exposure decreased collagen type I expression. **H:** mRNA levels of

Col1A1 increased 2-fold after combined exposure to 14 day Beta + 7 day LPS. 14 day LPS exposure followed by Beta treatment decreased *Col1A1* mRNA by 40%. **I:** *Col1A2* mRNA levels decreased in 14d LPS, 7d Beta and 14d LPS + 7d Beta lambs by 50%. * $p<0.05$ versus controls and § $p<0.05$ between experimental groups using a one-way ANOVA with Dunnett's post hoc test.

Discussion

LPS exposure leads to changes in Shh signaling in the fetal lung

In the context of developmental biology research, less is known about later fetal lung development than early organogenesis. Later lung development is, however, an area of human biology where clinical care interfaces with development since survival after very preterm birth at 60% of gestation is now frequent. We used an animal model with similarities to late gestation human lung development to test two very common clinical exposures, chorioamnionitis and antenatal steroids. We used 7 and 14 day intra-uterine periods of exposure based on our previous findings of a delay in alveolar development after 7 and 14 days of LPS-induced inflammation (25, 54). Here we show that fetal lung exposure to LPS-induced inflammation (29) is accompanied by changes in the Shh pathway, which is crucial for early lung development. In addition, we demonstrated that a maternal intra-muscular injection of betamethasone attenuated the effects of LPS on this developmental pathway. We therefore provide some molecular insights into the observational data from clinical practice that maternal corticosteroids are beneficial despite the inflammation of chorioamnionitis (20).

Exposure to intra-amniotic LPS has been shown to cause severe lung inflammation and damage leading to structural changes in the fetal lung which mimic pulmonary changes seen in BPD patients (26). Although in this study we did not measure an increased expression of HSP70, an indicator of oxidative-stress mediated lung damage, previous work from our group showed that LPS-induced chorioamnionitis causes fetal lung injury as early as 5h after the exposure (26). Tissue remodeling was further characterized by increased proliferation seen up to 14 days after the LPS exposure and maturation of alveolar type II cells (26, 29).

As evidence is accumulating that the Shh pathway is involved during aberrant lung development and disease (24, 51), we asked if intra-amniotic LPS exposure altered Shh signaling in the fetal lung. Recently, LPS was shown to down-regulate Shh in vitro in pulmonary microvascular endothelial cells (55). In our study, LPS strongly down-regulated mRNA levels of *Shh* and its signaling components *Gli1* and *Gli2* in the ovine fetal lung. LPS exposure also selectively decreased Gli1 protein expression in the distal epithelial tips where Shh signaling in the lung is mainly localized (57). Shh expression can be induced by retinoic acid (40), which is decreased in babies who develop BPD (44). The down-regulation of Shh is in line with a previous study from our group, in which LPS-induced chorioamnionitis

decreased retinoic acid in fetal sheep lungs (25). Therefore, the LPS-induced decrease of retinoic acid may have decreased *Shh* mRNA.

The decreases in mRNA and protein expression of Shh signaling pathway components were accompanied by 2- to 3-fold increases in *FGF10* and *BMP4* expression 14 days after LPS exposure, following a slight decrease 7 days after LPS exposure. The initial decrease in *FGF10* expression, which has also been measured in the lung tissue of infants with BPD (8), might be due to the activation of TLR2 and 4 by LPS, which can suppress FGF10 through binding of NF-κB to the FGF10 promotor (7). As FGF10 induces BMP4 expression in the developing lung (52), the inhibition of FGF10 may indirectly decrease BMP4 expression. The continuous suppression of *Shh* measured at 7 and 14 days after LPS exposure, which normally down-regulates FGF10 (6), may have caused FGF10 and BMP4 levels to rise.

The changes in these pivotal developmental pathways were accompanied by changes in mRNA levels and deposition of structural proteins which are known to direct alveolar septation (9, 25, 49). In a normally developing lung, focal expression of elastin identify sites for alveolar budding (13). In the lungs of the 7 day LPS exposed lambs, mRNA levels of *ELN* and the numbers of elastin foci decreased. Persistent exposure to LPS did not only result in less elastin foci but also increased collagen deposition along the alveolar wall. These observations of dysregulated elastin and collagen deposition in the fetal lung are consistent with ventilation-induced (1, 9, 12) and inflammation-induced (25, 27) animal models of BPD and histology reports of BPD patients (46, 48). Although Shh signaling has been implicated in the activation of fibroblasts and production of extracellular matrix (ECM) proteins such as collagen (19, 23, 51), Shh seems to act mainly through regulation of FGF10 to direct ECM deposition in the developing lung. Shh expression at the pulmonary epithelial tips controls FGF10 expression which in turn controls bud size and shape (37). Both overexpression and inhibition of FGFs lead to inhibition of lung branching and alterations in ECM protein expression (15, 38, 45).

Betamethasone and lung development

Antenatal corticosteroids are routinely administered to mothers who are at risk of preterm birth to mature the fetal organs (4). A secondary benefit may be suppression of inflammation (4). Antenatal steroids also reduce adverse neonatal outcome after preterm birth associated with chorioamnionitis (20), which constitute the majority of early gestational preterm births (4). The effect of these combined pro- and anti-inflammatory stimuli on pathways that are crucial for the developing lung are however unknown.

Recently, we showed that betamethasone treatment before the LPS exposure suppressed fetal lung inflammation by an unknown priming or conditioning mechanism of the fetal immune system (29). As such little pulmonary damage was inflicted by the exposure to LPS and no changes in the developmental pathways which we studied were detected. Very little

is known about the effect of maternal corticosteroids on Shh, FGF10 and BMP4. Corticosteroids can inhibit Shh-mediated neural development and as such can have a detrimental effect on the neonatal developing brain (22). We found that maternal corticosteroids alone did not change the expression of these factors in the fetal lung in comparison with controls. Moreover, the effects of LPS on these factors were neutralized by maternal betamethasone, irrespective if betamethasone was given 7 days before or after LPS.

At the lung structural level, corticosteroid treatment before LPS exposure could mitigate the decrease in elastin foci and increase in *ELN* mRNA levels. Corticosteroid post-treatment even increased elastin foci which is in line with previous reports showing that corticosteroids can stimulate tropoelastin production in a dose dependent manner (34, 39) most likely through at transforming growth factor (TGF)-β3 mediated mechanism (56). Here we show that corticosteroid treatment can counteract the negative effects of the LPS exposure on elastin deposition.

Furthermore, treatment with only betamethasone 7 days before delivery decreased *Col1A2* mRNA levels. Whether this resulted in a decreased collagen deposition in a later stage remains to be investigated. However, combined exposure to 7 day Beta treatment with 14 day LPS exposure did decrease collagen deposition. Several studies have shown beneficial effects of corticosteroid treatment on attenuating fibrotic processes following lung injury (17, 36, 50). Preterm infants at risk of BPD patients showed a significant reduction in markers of collagen synthesis after prenatal corticosteroid treatment up to 7 days after administration (18, 43). Surprisingly, corticosteroid treatment before the inflammatory stimulus stimulated collagen deposition although little pulmonary inflammation was noticed in these animals (29). Brenner et al. demonstrated in vitro that corticosteroids do not uniformly suppress the fibrotic activity of lung fibroblasts in vitro. This could explain our contradictory results and the diverse outcomes of lung morphology and function of BPD patients after corticosteroid treatment (11).

In this study, we modeled a common clinical exposure to provide new insights into the effects of exposures on fetal lung development (47, 54). However, there are limitations, as exposures at different time points, for different intervals and to a single dose or repeated doses of corticosteroids during fetal development may have different outcomes. The effects of pro- and anti-inflammatory stimuli on the developing lung are clearly complex. It remains to be determined whether the observed changes are the result of direct or indirect effects of LPS and betamethasone with these developmental pathways. Based on these results, intervention studies using inhibitors of the Shh pathway (e.g. cyclopamine (53)) would be helpful. The developing lung is plastic in that it is continually changing over gestation. Because chorioamnionitis is often clinically silent, the duration of exposure of the lung to chorioamnionitis and the subsequent inflammatory response are unknown. It is generally

unknown whether antenatal maternal corticosteroids are administered before or after the onset of chorioamnionitis (4).

In conclusion, this report shows that LPS-induced chorioamnionitis can disturb Shh signaling during early alveolar lung development, which is partially mitigated by betamethasone exposure. Therefore, this report provides some insights into the complicated interactions that can alter lung structure during the maturation phase of lung development where clinical intervention may already occur.

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Chapter 4

Altered canonical Wingless-Int (Wnt) signaling in the ovine fetal lung after exposure to intra-amniotic lipopolysaccharide (LPS) and antenatal betamethasone

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Abstract

Rationale Antenatal inflammation and maternal corticosteroids induce fetal lung maturation but interfere with late lung development. Canonical Wingless-Int (Wnt) signaling has an important role in directing lung development and repair. We recently showed that intra-amniotic lipopolysaccharide (LPS) exposure disrupted developmental signaling pathways in the lungs of preterm lambs. Therefore, we hypothesized that pulmonary Wnt signaling was altered by exposure to intra-amniotic LPS and/or antenatal corticosteroids.

Methods Ovine fetuses were exposed to intra-amniotic LPS, a maternal intra-muscular betamethasone injection, a control saline injection or a combination thereof at 107 and/or 114 days gestational age (GA) (term=150d GA) before delivery at 121d GA.

Results Exposure to intra-amniotic LPS decreased the lung expression of lymphoid enhancer-binding factor 1 (LEF1), a major Wnt pathway effector. *WNT1*, *WNT4* and downstream messenger β-catenin were decreased after LPS exposure. *WNT7b* mRNA increased 4-fold 14d post LPS exposure. Betamethasone treatment 7d before or after LPS exposure attenuated most changes in Wnt signaling.

Conclusion Intra-amniotic LPS exposure decreased canonical Wnt signaling in the developing lung. Maternal corticosteroids normalized Wnt signaling in the fetal lung. This study provides new insights into possible mechanisms by which prenatal inflammation affects lung development and how corticosteroid administration can be beneficial in this setting.

Introduction

Preterm birth is frequently initiated by chorioamnionitis, an intra-uterine inflammation of the chorio-amniotic membranes (2). Exposure to antenatal inflammation may contribute to adverse neonatal outcomes in preterm infants including lung injury (9, 14). As a result, bronchopulmonary dysplasia (BPD) can develop, which is characterized by fewer and larger alveoli due to an arrested development of alveolar septation and impaired pulmonary microvascular development (13). The corticosteroid betamethasone is routinely administered to mothers at risk for preterm delivery to improve neonatal survival (25). Although corticosteroids accelerate lung maturation, they also inhibit the outgrowth of secondary septa and therefore can interfere with late lung development (23).

Wingless-Int (Wnt) signaling plays a role in lung development by regulating epithelial and mesenchymal interactions in an autocrine and paracrine manner (17, 36). The canonical Wnt pathway signals through β-catenin (36). In the absence of Wnt ligands, β-catenin is phosphorylated by glycogen synthase kinase (GSK)-3β, ubiquitinated and degraded (38). Upon activation of the Wnt cascade, GSK-3β becomes phosphorylated and inhibited (24). Consequently, β-catenin phosphorylation is prevented and the amount of cytosolic β-catenin increases (24). The accumulated β-catenin translocates to the nucleus and regulates gene transcription via formation of an active transcription complex with T cell-specific transcription factor (TCF)/lymphoid enhancer-binding factor (LEF) family members (33). The importance of Wnt signaling during lung development was demonstrated in several transgenic models of different Wnt isoforms. $Wnt2^{-/-}$ mice have severe lung hypoplasia and a poorly developed lung mesenchyme at birth (10). Similarly, knockout of Wnt7b in mice results in lung hypoplasia and perinatal death due to respiratory failure (28). Reduced pulmonary Wnt7b expression impairs mesenchymal growth and vascular development (28). Wnt1 and Wnt4 are associated with the expression of matrix metalloproteinases, which are known to be involved in lung epithelial repair processes and pulmonary fibrosis (27, 40).

Previously we showed that exposure to intra-amniotic lipopolysaccharide (LPS) induced pulmonary inflammation in fetal lambs and influenced lung developmental pathways (4, 5). Intra-amniotic LPS exposure altered the expression of the lung structural proteins elastin and collagen leading to a simplified lung structure as seen in BPD patients (32). Betamethasone pre- or post-treatment partially counteracted these effects (5). However, little is known about the molecular signaling cascades by which perinatal events affect late lung development. Rodent models of neonatal hyperoxia-induced lung injury have recently related perturbations in the Wnt cascade with developmental alterations in postnatal lung development, similar to BPD (6). In this study, we investigated the effects of intra-uterine inflammation which is implicated in the pathogenesis of BPD, on canonical Wnt signaling in a preterm ovine model in which the pulmonary development closely resembles the early alveolarization stage in extremely preterm infants (37). We hypothesized that Wnt signaling was altered in ovine fetal lungs exposed to *in utero* inflammation and/or antenatal

corticosteroids. For this purpose, we examined different Wnt isoforms and downstream effectors of the canonical Wnt pathway in the lungs of fetal sheep that were exposed to intra-amniotic LPS and/or antenatal corticosteroids.

Materials and Methods

Animal model and sampling protocol

All animal experiments were approved by the Animal Ethics Committees of Cincinnati Children's Hospital Medical Center and The University of Western Australia (animal ethics protocol RA/3/100/830). Time-mated Merino ewes with singleton fetuses were randomly divided into six treatment groups (n=5-8 per group). The ewes received either: an intra-amniotic (IA) injection of 10 mg LPS (*Escherichia Coli*, 055:B5 Sigma-Aldrich, St. Louis, MO, USA), an intra-muscular injection of the corticosteroid betamethasone (Celestone Soluspan 0.5 mg/kg maternal weight, Schering-Plough, North Ryde, New South Wales, Australia), a control saline injection or a combination thereof at 107 and/or 114 days gestational age (dGA) (Figure 1).

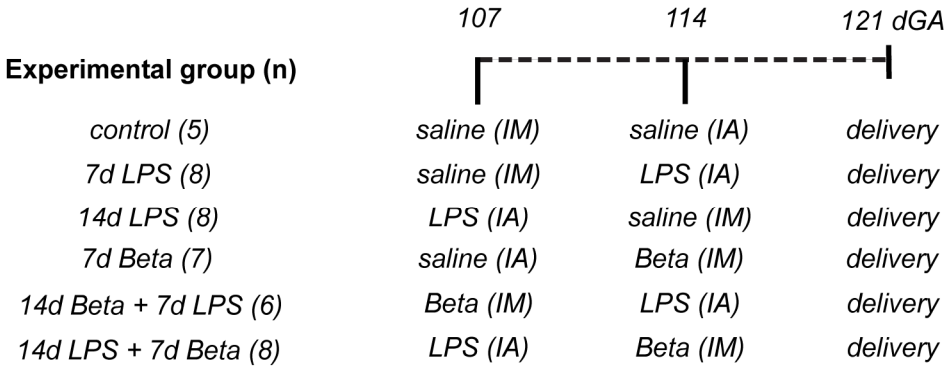

Figure 1: Study design. Ovine fetuses were exposed to intra-amniotic (IA) LPS, a maternal intra-muscular (IM) betamethasone (Beta) injection, a control saline injection or a combination thereof at 107 and/or 114 days gestational age (GA) (term=150d GA). Lambs were delivered preterm by caesarean section at 121d GA.

Since betamethasone treatment can induce preterm delivery in sheep, all ewes also received a single intra-muscular injection of medroxyprogesterone acetate (Depo-Provera 150 mg, Kenral, New South Wales, Australia) at 100 dGA to decrease the risk of preterm labor (21). Lambs were surgically delivered preterm at 121 dGA (term is 150 dGA) and were humanely euthanized. The lungs were removed and samples of right lower lobe (RLL) tissue were snap frozen. The whole right upper lobe (RUL) was inflation fixed for 24h in 10% buffered formalin at a pressure of 30 cm H_2O and processed for paraffin embedding.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from snap frozen RLL tissue using the SV Total RNA Isolation System (Z3100, Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. A DNase treatment was performed to eliminate possible genomic DNA contamination, using the RQ1 RNase-Free DNase kit (M6101, Promega Corporation). cDNA was synthesized with a Transcriptor First Strand cDNA Synthesis Kit using Anchored-oligo(dT)18 Primers (04379012001, Roche Applied Science, Penzberg, Germany), according to the manufacturer's protocol. Primers were created based on genome sequences of *Bos taurus* and *Ovis aries* (Table 1). Dilution experiments were performed to ensure proper PCR amplification efficiency of the primers. qPCR reactions were run in duplicate for each primer set at the appropriate melting temperature with a LightCycler 480 Instrument (Roche Applied Science) and LightCycler 480 SYBR Green I Master mix (4707516001, Roche Applied Science). qPCR results were normalized to the housekeeping gene ovine ribosomal protein S15 (ovRPS15). Mean fold changes in mRNA expression, compared to control animals, were calculated using the ΔΔCt-method (20).

Table 1: Primers for qPCR

Gene		Sequence (5`-3`)	Amplicon size	T^m
WNT1	Fw	ATTTATCTTCGCCATCACCTC	123bp	64°C
	Rv	ATTCGATGGAGCCCTCTG		
WNT2	Fw	GGTGGCTGCAGTGATAACATTGAC	60bp	58°C
	Rv	ACCTCTTTACAGCCTTCCTGCC		
WNT4	Fw	GCTGGGCTCCAAGTACACC	241bp	60°C
	Rv	GGCTATCCTGACACACATGC		
WNT7b	Fw	TGCACTCCAGCTTCATGCGC	60bp	58°C
	Rv	ACCTGCACAACAACGAGGCG		
CTNNB1	Fw	CTATTGAAGCCGAGGGAG	200bp	60°C
	Rv	CAAGATCAGCAGTCTCATTCC		
GSK3B	Fw	CGAGACACACCTGCACTCTT	157bp	60°C
	Rv	CACGGTCTCCAGCATTAGCA		
LEF1	Fw	CAACTCCAAACAAGGCATGTCC	90bp	60°C
	Rv	GGTAATCTGTCCAACACCACC		
ovRPS15	Fw	CGAGATGGTGGGCAGCAT	93bp	60°C
	Rv	GCTTGATTTCCACCTGGTTGA		

Immunohistochemistry

Paraffin-embedded RUL lung tissue sections (4 µm) were deparaffinized using a xylene and ethanol (100%, 96%, 70%) series. Incubation in sub-boiling citrate buffer (10 mM, pH 6.0) for 30 minutes was used for antigen retrieval. To block endogenous peroxidase activity, slides were incubated in phosphate buffered saline (PBS, pH 7.4) with 0.5% hydrogen peroxide (H_2O_2) for 20 minutes. Nonspecific binding of antibodies was prevented by incubating the sections with 10% normal goat serum (NGS) in PBS. Sections were stained overnight at 4°C

with monoclonal rabbit anti-LEF1 (1:100, 2230, Cell Signaling). After 1h of incubation with biotinylated polyclonal swine anti-rabbit (1:200, E0353, Dako, Glostrup, Denmark) secondary antibody, the immunostaining was augmented with the addition of a peroxidase-labeled ABC complex (1:500; Vectastain Elite ABC kit PK-6200, Vector Laboratories, Burlingame, CA, USA). Immunostaining was visualized with Nickel-enhanced diaminobenzidine (NiDAB) and the sections were counterstained with 0.1% Nuclear Fast Red.

Staining of the sections was evaluated by light microscopy (DM2000, Leica Microsystems, Wetzlar, Germany). The area fraction (%) of LEF1 immuno-reactivity was measured in six representative images per section by applying a standard threshold using a specifically designed algorithm in Leica QWin Pro V 3.5.1 software (Leica, Rijswijk, the Netherlands) and corrected for the total percentage of lung tissue measured.

Western blot

For protein analysis snap-frozen RLL tissue was homogenized in ice-cold radio-immunoprecipitation assay buffer (R0278, Sigma-Aldrich) supplemented with 0.1% protease inhibitor cocktail (P9599, Sigma-Aldrich). Homogenates were centrifuged for 5 minutes at 12x relative centrifugal force. To determine protein concentrations, a Micro Bicinchoninic Acid Protein Assay kit (23235, Thermo Fisher Scientific Inc, Waltham, MA, USA) was used with bovine serum albumin as a standard, according to the manufacturer's instructions. Samples were diluted in 5x sample buffer (consisting of glycerol, sodium dodecyl sulphate (SDS), 0.5M Tris-HCl pH 6.8 + 0.4% SDS, bromophenol blue, dithiothreitol and Milli-Q) and denatured by heating at 95°C for 10 minutes. Equal amounts of protein (40 µg per sample) were loaded on SDS-polyacrylamide gels. Proteins were separated within 1.5h (90V) by gel electrophoresis and transferred onto 0.2 µm pore size Protran BA83 nitrocellulose membranes (10402495, Whatman GmbH, Dassel, Germany) within 1h (350 mA).

Membranes were blocked for 1h with a 1:1 mixture of ODYSSEY Infrared Imaging System Blocking Buffer (927-40000, LI-COR, Lincoln, Nebraska, USA) and Tris buffered saline (TBS, 2.5 mM, pH 7.5) and incubated overnight at 4°C with the following primary antibodies: monoclonal rabbit anti-β-catenin (1:1000; 9582, Cell Signaling Technology, Danvers, MA, USA), monoclonal rabbit anti-GSK-3β (1:4000; 9315, Cell Signaling) or monoclonal mouse anti-β-actin (1:1000; A5441, Sigma-Aldrich). IRDye 800CW conjugated goat anti-rabbit (1:6000; 926-32211, LI-COR) or IRDye 680RD conjugated donkey anti-mouse (1:6000; 926-32222, LI-COR) were used as secondary antibodies (1h). Protein bands were detected and analyzed using a LI-COR Odyssey Infrared Imager system. Results were normalized to the housekeeping gene β-actin.

Data analysis

Statistical analysis was conducted using GraphPad Prism v5.0 software. One-way analysis of variance (ANOVA) with Tukey's test for post-hoc analysis was used to compare the groups. All data are presented as means ± standard error of mean (SEM). $p<0.05$ was considered to be significant.

Results

Pulmonary LEF1 expression

In order to investigate changes in pulmonary Wnt signaling, we determined the mRNA levels and immuno-reactivity of LEF1, the major downstream effector of the Wnt pathway (7). Exposure to LPS 7 or 14 days before preterm delivery resulted in decreased *LEF1* mRNA levels (Figure 2A) and LEF1 protein immuno-reactivity (Figure 2B). Betamethasone (Beta) treatment before the LPS exposure but not after LPS exposure prevented the decrease in LEF1. Representative images of LEF1 staining are shown for controls (Figure 2C) and 14 day LPS exposed lungs (Figure 2D).

Lung mRNA levels of WNT isoforms

The mRNA expression of different WNT isoforms was measured to characterize the upstream changes in Wnt signaling after intra-amniotic LPS exposure. *WNT1* mRNA levels in the fetal lung decreased by 90% 7 days after the exposure to LPS compared to controls (Figure 3A). Exposure to LPS at 14 days followed by betamethasone exposure at 7 days before delivery increased *WNT1* mRNA levels when compared to 14 day LPS exposure alone. *WNT2* mRNA decreased by 80% 14 days after LPS exposure irrespective of betamethasone post-treatment (Figure 3B). *WNT4* mRNA levels were significantly decreased in all LPS-exposed treatment groups compared to controls (Figure 3C). In contrast, *WNT7b* levels increased 4-fold 14 days after LPS exposure compared to controls (Figure 3D). Treatment with betamethasone 7 days after the LPS exposure normalized this increase. However, in this group there was still a trend towards increased *WNT7b* expression.

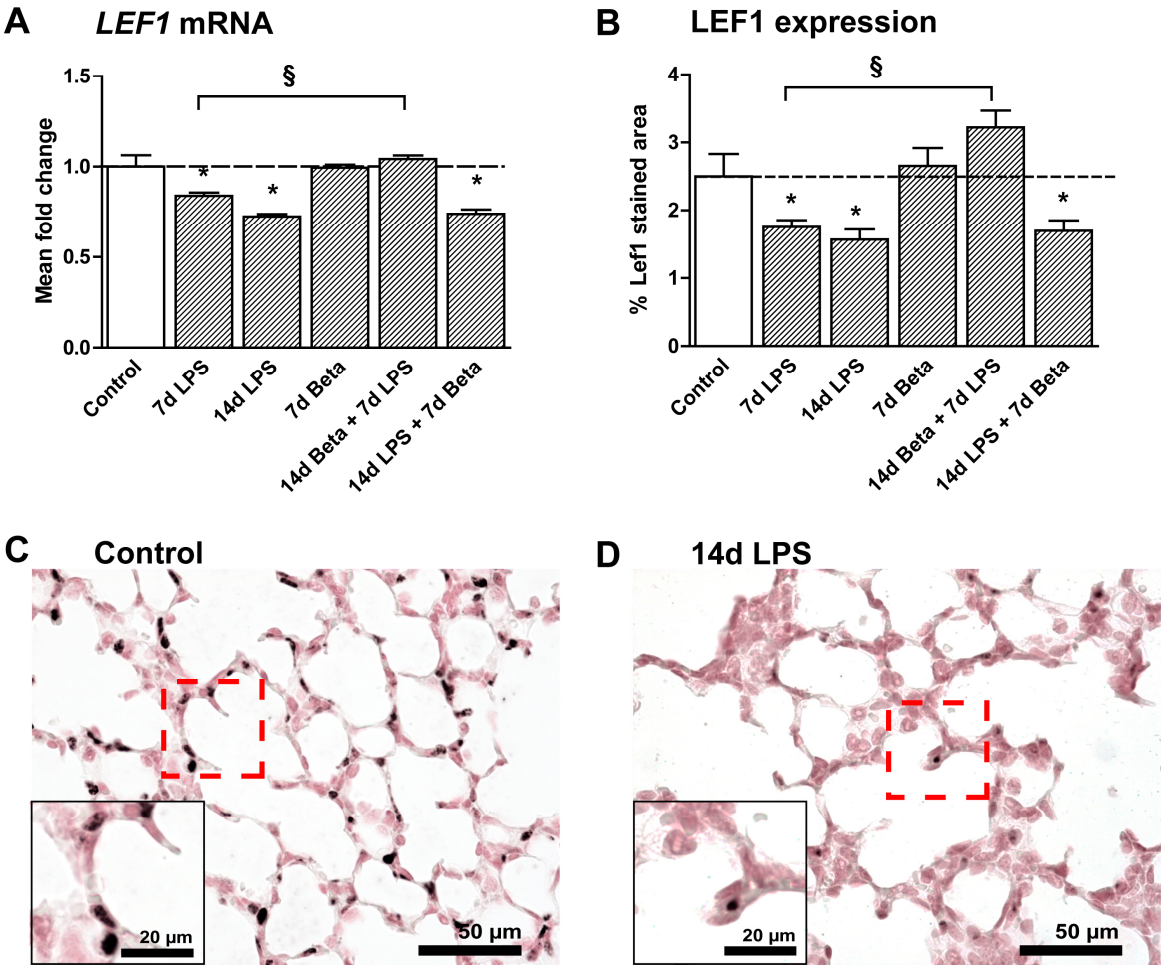

Figure 2: Pulmonary LEF1 expression. **A:** mRNA levels of *LEF1* were decreased 7 and 14 days after LPS exposure. Combined exposure to 14 day LPS and 7 day Beta also resulted in decreased *LEF1* mRNA levels. **B:** The area fraction (%) of LEF1 immuno-reactivity in the lung decreased after exposure to LPS 7 or 14 days before delivery. Beta treatment before the LPS exposure but not after LPS exposure prevented the decrease in LEF1. LEF1 staining in the fetal lung as seen in control animals (**C**) and 14d LPS exposed animals (**D**). *p<0.05 versus controls and § p<0.05 between experimental groups using a one-way ANOVA with Tukey's post hoc test.

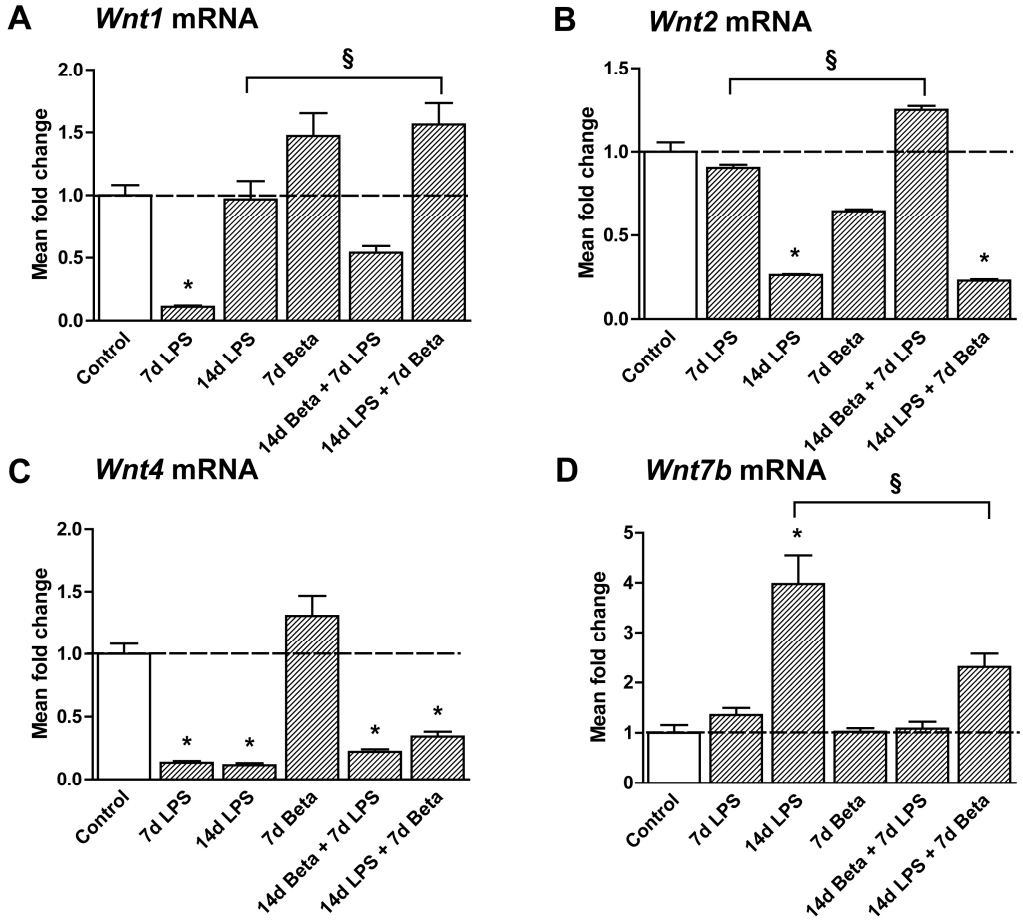

Figure 3: *Lung mRNA levels of WNT isoforms.* **A:** The mRNA levels of *WNT1* decreased 9-fold 7 days after LPS exposure compared to controls. **B:** *WNT2* mRNA decreased after 14 days of LPS exposure irrespective of the betamethasone treatment. **C:** Levels of *WNT4* mRNA decreased in all LPS exposed groups compared to controls. **D:** mRNA levels of *WNT7b* increased nearly 4-fold 14 days after LPS exposure compared to controls. This increase was prevented if betamethasone (Beta) was administered 7 days after LPS exposure. *p<0.05 versus controls and § p<0.05 between experimental groups using a one-way ANOVA with Tukey's post hoc test.

β-catenin and GSK-3β expression in the fetal lung

LPS and/or betamethasone exposure did not change *CTNNB1* mRNA or *GSK3B* mRNA levels in the fetal lung (data not shown). β-catenin protein expression decreased 7 days after the exposure to LPS and after exposure to LPS followed by betamethasone post-treatment (Figure 4A). GSK-3β protein expression in the fetal lung did not change significantly in any of the experimental groups (Figure 4B).

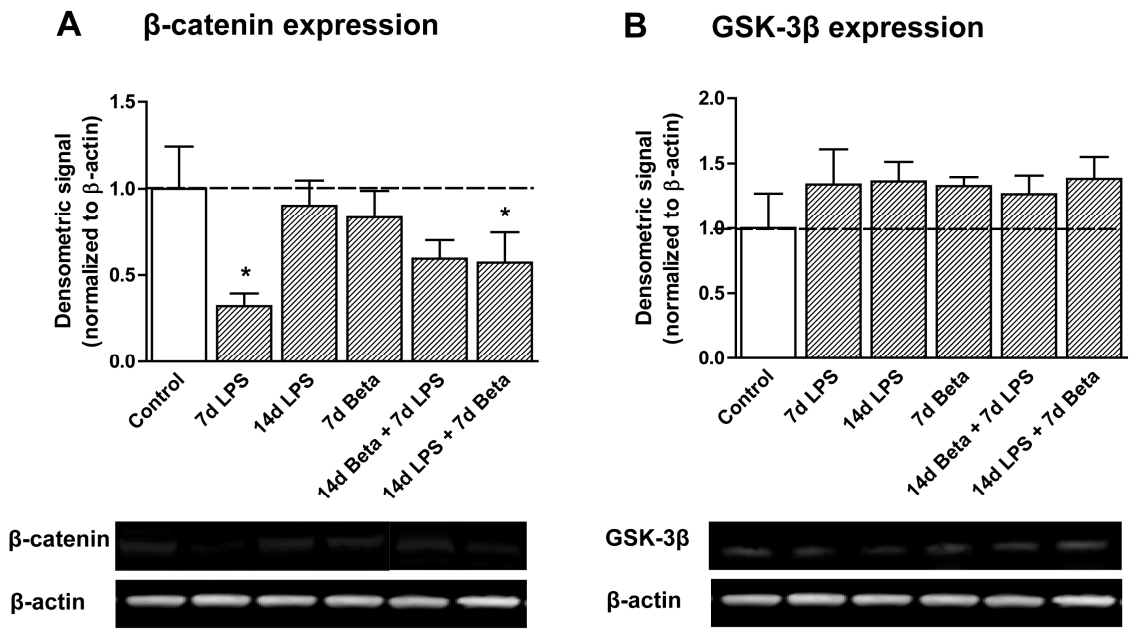

Figure 4: β-catenin and GSK-3β expression in the fetal lung**. A:** β-catenin protein expression decreased by two-thirds in fetal lungs exposed to LPS for 7 days and in lung exposed to 14 day LPS + 7 day Beta. **B:** GSK-3β protein expression did not significantly change in any of the experimental groups. * $p<0.05$ versus controls using a one-way ANOVA with Tukey's post hoc test.

Discussion

Although epidemiological and experimental studies have demonstrated a clear correlation between prenatal inflammation and altered fetal lung development (1, 18), little is known about the molecular pathways mediating these effects. We found that exposure to intra-amniotic LPS down regulated the Wnt pathway as illustrated by decreased nuclear LEF1 immuno-reactivity and decreased β-catenin protein expression. β-catenin signaling during pulmonary development is necessary for growth and differentiation of pulmonary epithelial cells (22). Subsequently, inhibition of Wnt signaling can lead to impaired branching and defects in vascular development in the lung (28, 31). Previously we demonstrated reduced alveolar septation following LPS exposure in this animal model (5). Now, we show aberrant Wnt signaling in this model of disrupted lung morphology after exposure to intra-amniotic inflammation.

Of the four WNT isoforms we measured, *WNT1* and *WNT4* had similar patterns of decreases as LEF1 after intra-amniotic LPS exposure suggesting that these changes are responsible for the decreased LEF1 expression. However, differential expression patterns of these WNT isoforms and others occur both in lung development and in lung disease (16, 39). The net outcome of these changes is dependent on not only the Wnt receptor and the affinity of the Wnt isoform for that receptor (3, 15), but also on the cross-talk of Wnt components with other signaling pathways such as the TGF-β pathway (4, 11). Interestingly, 14 days after the

exposure to LPS, *WNT7b* mRNA levels were up regulated. These relatively late effects may represent a response of the developing lung to repair the damage from intra-amniotic LPS induced inflammation. Wnt signaling can stimulate tissue remodeling, cell migration and wound healing in the lung (26). Activation of the Wnt pathway in alveolar type II cells promotes epithelial survival and differentiation towards alveolar epithelial cells after lung injury (8). β-catenin can induce fibroblast growth factor (FGF)10 expression in para-bronchial smooth muscle cell progenitors which help to repair the damaged lung epithelium (35). We previously showed in this animal model that exposure to intra-amniotic LPS was accompanied by increased levels of FGF10 in the fetal lung 14 days after the injection (5). Canonical Wnt signaling may stimulate FGF10 expression and subsequent myofibroblast differentiation in the ovine fetal lung after exposure to intra-amniotic inflammation. Although canonical Wnt signaling plays a key role in the repair phase after lung injury, aberrant activation of the Wnt pathway can have deleterious consequences. Increased canonical Wnt signaling is seen in fibrotic diseases such as idiopathic fibrosis and ventilation induced lung injury (16, 34). It however remains to be determined if Wnt signaling remains perturbed in this model, and if so, how persistently altered Wnt signaling affects lung development beyond the fetal environment.

Chorioamnionitis and antenatal corticosteroids are common exposures for the preterm fetus. Although they both influence fetal lung maturation and development, the interaction between both exposures is less understood. We previously reported that corticosteroids given 7 days before the exposure to intra-amniotic LPS inhibited pulmonary inflammation and injury in the ovine fetal lung (19). Here, we further demonstrate that corticosteroid treatment before the exposure to LPS attenuated the down regulation in Wnt signaling. A single dose of antenatal corticosteroid 7 days after the LPS exposure also normalized Wnt signaling in the fetal lung. Others have reported interactions that are consistent with these results. Stimulation of the glucocorticoid receptor (GR) can activate GSK-3β which in turn increases β-catenin breakdown and thus inhibits the Wnt pathway (29). In addition, the GR can directly decrease Wnt signaling by binding to the TCF-β-catenin complex (30).

This is the first report to show altered Wnt signaling after antenatal inflammation in the fetal lung. However, future in vitro and in vivo studies are needed to demonstrate a causal link between the presence of inflammation and disturbances in this pathway and subsequently late lung development. Although we were not able to perform an in-depth analysis of the investigated pathway due to lack of specific reagents, these initial observations of changes in Wnt signaling do provide interesting options for possible intervention strategies since re-directing Wnt signaling in the fetal lungs may provide beneficial outcomes for the premature lung. Inhibition of GSK3-β by a pharmacological modulator SB216763 resulted in reduced pulmonary inflammation and improved alveolarization in a hyperoxia rodent model of BPD (12). Future studies will be needed to show if and how pharmacological modulation of the Wnt pathway can re-direct late lung development after exposure to chorioamnionitis. In

conclusion, our results demonstrate that fetal lung exposure to LPS can decrease Wnt signaling and that maternal corticosteroid administration can partially prevent these changes. Thereby this study helps to provide insight into the beneficial effects of antenatal corticosteroid treatment on the fetal lung in the setting of intra-uterine inflammation.

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CHAPTER 5

Intra-amniotic lipopolysaccharide exposure changes cell populations and structure of the ovine fetal thymus

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Abstract

Rationale Chorioamnionitis induces preterm delivery and acute involution of the fetal thymus which is associated with postnatal inflammatory disorders. We studied the immune response, cell composition and architecture of the fetal thymus following intra-amniotic lipopolysaccharide (LPS) exposure.

Methods Time-mated ewes received an intra-amniotic injection of LPS 5, 12 or 24h or, 2, 4, 8 or 15d before delivery at 125d gestational age (term=150d).

Results LPS exposure resulted in decreased blood lymphocytes within 5h and decreased thymic cortico-medullary ratio within 24h. Thymic *IL6* and *IL17* mRNA increased 5-fold 24h post-LPS exposure. Increased *TLR4* mRNA and NF-κB positive cells at 24h after LPS delivery demonstrated acute thymic activation. *TLR4* and *IL1* mRNA increased by 5-fold and the number of Foxp3-positive cells decreased 15d after exposure.

Conclusion Intra-amniotic LPS exposure caused a pro-inflammatory response, involution and a persistent depletion of thymic Foxp3+cells indicating disturbance of the fetal immune homeostasis.

Introduction

Chorioamnionitis, an intra-amniotic inflammation of the chorio-amniotic membranes documented by histologic findings or culture, is commonly associated with preterm birth and adverse neonatal outcomes (15, 17). Clinical chorioamnionitis can manifest itself with maternal fever, uterine tenderness and maternal and fetal tachycardia (19, 41). However, chorioamnionitis is often a clinically silent infection without maternal symptoms referred to as histological chorioamnionitis (16). By histological examination of the placenta, the diagnosis of chorioamnionitis can be subdivided into acute of chronic depending on the infiltration of respectively neutrophils or lymphocytes (27, 37).

Intra-uterine exposure to infection or inflammation can have profound effects on fetal lung, gut and brain development, thereby increasing the risk for respiratory, gastrointestinal and neurodevelopmental complications(4, 21, 40, 44). Therefore, chorioamnionitis has been characterized as a 'multi-organ disease of the fetus' (12).

Although the systemic inflammatory response of the fetus after intra-uterine inflammation has been the focus of ongoing studies, the effects on the inflammatory organs such as the thymus, spleen and lymph nodes has barely been studied. Clinically chorioamnionitis is however associated with a decreased thymus size in very low birth weight preterm infants (10, 52). De Felice et al. demonstrated that preterm neonates exposed to subclinical chorioamnionitis had smaller thymuses at birth on chest radiographs than preterm neonates without chorioamnionitis (9). Histological examination at autopsy showed severe morphological changes of the fetal thymus with a reduced cortico-medullary (C/M) ratio and low thymocytes count when fetuses were exposed to intra-uterine infection (47). However, these severe changes may not represent the thymic changes that occur in the majority of surviving fetuses exposed to chorioamnionitis.

A small thymus size in preterm infants is a strong indicator for other adverse outcomes such as bronchopulmonary dysplasia (7, 8, 42) and fetal inflammatory response syndrome (11). Thymic involution in preterm infants is associated with a higher risk for the development of cerebral white matter damage, the major antecedent of cerebral palsy (32). Moreover, a small thymus size at birth can lead to a smaller T-cell repertoire and lower thymic output leading to enhanced vulnerability to infections in later life (1, 13). Thymic involution also correlated with the frequency of infections in preterm infants admitted to the neonatal intensive care unit (23, 24), with the grade of thymic involution being related to the duration of the illness (14). Although there is a growing body of clinical evidence suggesting that intra-uterine infection has profound effects on the fetal and neonatal immune system and the thymus in particular, little is known about the mechanisms by which exposure to *in utero* infection/inflammation affects the fetal thymus and the consequences of these changes on the function and maturation of the immune system. Therefore, we characterized the cellular and structural changes in the fetal thymus after exposure to intra-amniotic inflammation.

We used a preterm lamb model to evaluate the changes in the fetal thymus in a time related manner after intra-amniotic lipopolysaccharide (LPS) injection (25).

Materials and methods

Animal study

All studies were approved by the Animal Ethics Committees at The University of Western Australia and Cincinnati Children`s Hospital Medical Center. Time-mated ewes with singleton fetuses received a single intra-amniotic injection of 10 mg LPS (bolus of 10 mg *Escherichia Coli* 055:B5, Sigma Chemical, St. Louis, USA, dissolved in saline) resulting in an inflammatory response of the chorio-amniotic membranes and systemic inflammation of the fetus(31), 5h, 12h, 24h, 2d, 4d, 8d or 15d before preterm delivery (n=6-8) (Table 1). No differences were observed between control animals that received saline injections at different time points before delivery and were therefore combined in one control group. All lambs were delivered by cesarean section at 125 days gestational age (GA) (term = 150 days GA) which corresponds with 28-32 weeks in humans (50), a critical period of late development when most preterm infants are born with serious complications. Preterm lambs were euthanized after birth. Cord blood was used for white blood cell counts to measure systemic inflammation. Total white blood cell counts were performed with correction for nucleated RBC and differential counts were performed by a single blinded observer. The thymus was collected and tissue was snap frozen or fixed in 10% buffered-formalin for 24 hours.

Immunohistochemistry

Paraffin embedded thymic sections (4 µm, transverse) were stained for CD3 (A0452, DAKO, Glostrup, Denmark), Foxp3 (14-7979, eBiosciences, San Diego USA), cleaved caspase-3 (Asp175, #9661S, Cell Signaling Technology, Boston, USA), Ki67 (M7240, DAKO, Glostrup, Denmark) and RelB (component of NF-κB, sc-266, Santa Cruz Biotechnology, Santa Cruz, USA). The sections were deparaffinized and rehydrated in ethanol. Endogenous peroxidase-activity was blocked by incubation with 0,3% H_2O_2 in phosphate buffered saline (PBS, pH 7.4) (for CD3 and Foxp3) or in methanol (for cleaved caspase-3, Ki67 and RelB). Antigen retrieval was performed by incubating the sections in heated citrate buffer (10 mM, pH 6.0) for 30 minutes. Non-specific binding was prevented by incubating the slides for 30 minutes with 5% bovine serum albumin (BSA) (CD3), 20% normal goat serum (NGS) (Foxp3 and RelB) or 5% NGS (Ki67). This step was omitted for cleaved caspase-3. Slides were incubated overnight at 4°C with the diluted primary antibody (CD3 1:200, Foxp3 1:30, cleaved caspase-3 1:200, Ki67 1:50, RelB 1:2000) followed by 1h incubation at room temperature with the appropriate secondary biotin labeled antibody (swine-α-rabbit 1:500, E0353; goat-α-mouse 1:500, E0433, DAKO, Glostrup, Denmark). The immunostaining was enhanced with Vectastain ABC peroxidase Elite kit (PK-6200, Vector Laboratories, Burlingame, USA) followed by nickel sulfate-diaminobenzidine (NiDAB). Sections were counterstained with 0.1% Nuclear Fast Red. Evaluation was performed by light microscopy (Axioskop 40, Zeiss, Oberkochen, Germany) with LeicaQWin Pro v.3.4.0 software (Leica Microsystems, Wetzlar, Germany).

Three images in different lobes of the thymus that were representative for the CD3 and Ki67 staining were taken at 200x magnification. The percentage of area with CD3 and Ki67 positive staining was measured in these images using Image J software (Rasband, W.S., Image J US National Institutes of Health, Bethesda, USA). For the Foxp3, cleaved caspase-3 and RelB staining, positive cells were counted and the average numbers of 3 different high power fields of different lobes at a 200x magnification were given.

The morphology of the thymus was evaluated by light microscopy after hematoxylin and eosin staining. The cortico-medullary (C/M) ratio was quantified in three representative sections at 2.5x magnification using Image J software (Rasband, W.S., Image J US National Institutes of Health, Bethesda, USA).

RNA extraction and quantitative real-time PCR

Quantitative real-time PCR was performed to measure mRNA levels of *Toll-like receptor (TLR)2* and *4* which are the main recognition receptors for gram positive and negative bacteria*, interleukin (IL)1*, *IL6*, *IL10*, *IL17* and *interferon gamma (IFNγ)* which are important cytokines produced in response to inflammation(2). Total RNA was isolated from thymic tissue by Trizol/chloroform extraction and treated with RQ1 DNase (M610A, Promega, Madison, USA). The digestion of genomic DNA was confirmed by PCR using specific GAPDH primers. For the analysis of gene expression, RNA was converted to cDNA with the Transcription First Strand cDNA synthesis kit (Roche-Applied, Mannheim, Germany) according to manufacturer`s instructions using anchored oligo-primers. RT-PCR reactions were performed in duplicate with the LightCycler 480 SYBR Green I Master mix (Roche-Applied, Mannheim, Germany) on a LightCycler 480 Instrument according to the manufacturer's instructions with primers based on ovine specific sequences (Table 2). RT-PCR results were normalized to ovine 40S ribosomal protein S15 (ovRPS15) and mean fold changes in mRNA expression were calculated by the ΔΔCt-method(38).

Data analysis

Results are given as means ± standard error of means (SEM). Groups were compared using one-way ANOVA with Dunnett's for post-hoc analysis or by a non-parametric Kruskal-Wallis test as appropriate. Statistical analysis was performed by GraphPad Prism v5.0. Significance was accepted at $p<0.05$.

Table 2: Primers used for RT-PCR

Gene		Sequence (5`-3`)	Amplicon size	T^m
TLR2	Fw	GGCTGTAATCAGCGTGTTCA	160bp	64°C
	Rv	GATCTCGTTGTCGGACAGGT		
TLR4	Fw	GAGAAGACTCAGAAAAGCCTTGCT	200bp	65°C
	Rv	GCGGGTTGGTTTCTGCAT		
IL1	Fw	CACTGCCAGAAAATAAGCTGAAAC	79bp	63°C
	Rv	TGATCAAGCAAATCGCCTGAT		
IL6	Fw	ACATCGTCGACAAAATCTCTGCAA	90bp	65°C

	Rv	GCCAGTGTCTCCTTGCTGTTT		
IL10	Fw	CATGGGCCTGACATCAAGGA	102bp	64°C
	Rv	CGGAGGGTCTTCAGCTTCTC		
IL17	Fw	TGTGAGGGTCAACCTGAACAT	110bp	62°C
	Rv	TGATAATCGGTGGGCCTTCTG		
IFNγ	Fw	TCAAGCAAGACATGTTTCAGAAGTTCT	65bp	60°C
	Rv	CCGGAATTTGAATCAGCCTTTTGAA		
ovRPS15	Fw	CGAGATGGTGGGCAGCAT	93bp	60°C
	Rv	GCTTGATTTCCACCTGGTTGA		

Results

Systemic inflammation

The percentage of neutrophils and monocytes increased significantly in cord blood at 8d and 4d after intra-amniotic LPS exposure respectively (Table 1). The percentage of lymphocytes in cord blood decreased within 5h after LPS exposure and remained low until 2d post LPS exposure.

Table 1: Differential cell counts in the cord blood.

	Control *(n=8)*	***5h*** *(n=6)*	***12h*** *(n=7)*	***24h*** *(n=7)*	***2d*** *(n=6)*	***4d*** *(n=6)*	***8d*** *(n=7)*	***15d*** *(n=7)*
Lymphocytes $(x10^9/L)$	2.6±0.2	1.4±0.2*	1.4±0.2*	1.1±0.1*	1.7±0.3	2.9±0.5	3.1±0.1	2.1±0.2
Neutrophils $(x10^8/L)$	4.1±0.3	4.4±0.8	3.2±1.1	1.6±0.4	1.6±0.5	9.1±1.4	20.1±5.0*	10.6±4.9
Monocytes $(x10^8/L)$	1.5±0.3	0.7±0.3	1.3±0.2	0.4±0.1	1.2±0.5	4.1±0.7*	3.2±0.8	2.6±0.9

Data expressed as mean ± SEM. * $p<0.05$ versus controls

Thymic cortico-medullary ratio

Representative images for the thymic cortico-medullary (C/M) ratio are shown for controls (Figure 1A) and 8d LPS exposed animals (Figure 1B). The C/M ratio decreased significantly 24h and 8d after LPS exposure compared to controls (Figure 1C).

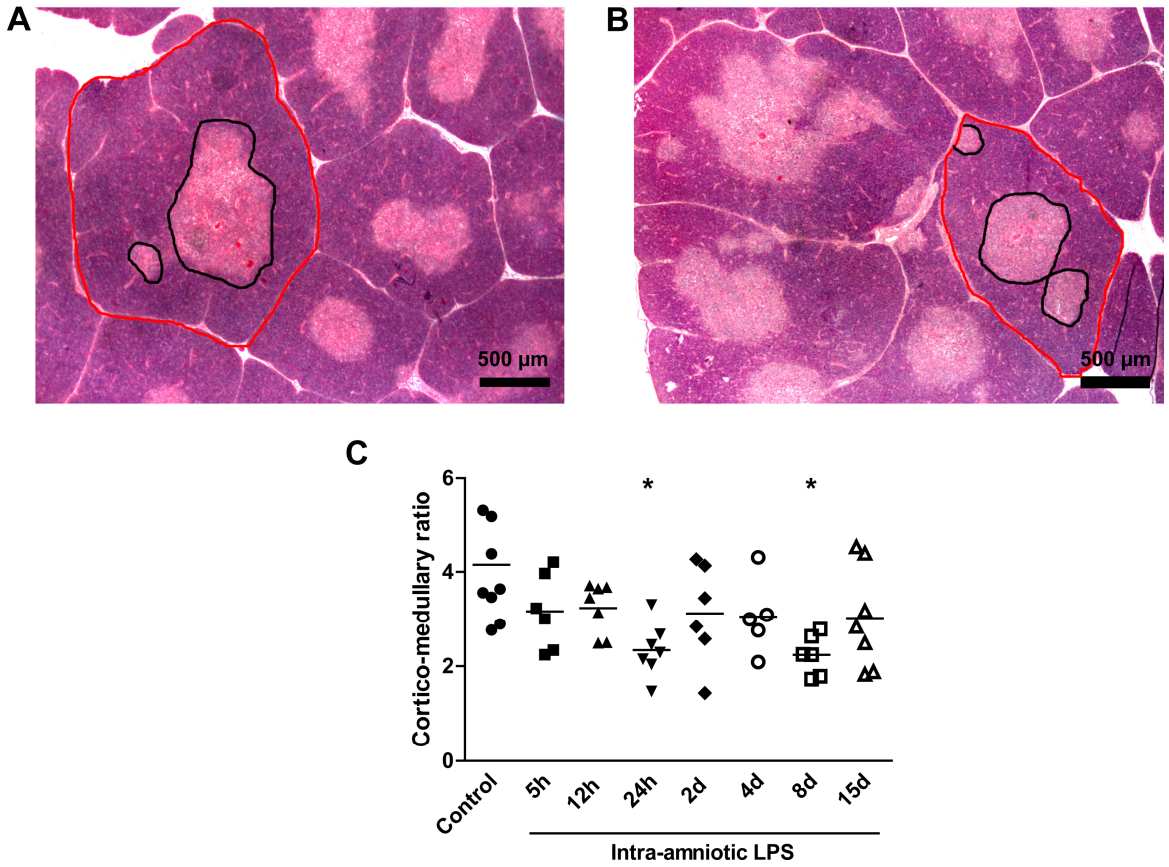

Figure 1: Cortico-medullary ratio. The cortico-medullary (C/M) ratio of the thymus was analyzed in H&E sections. Representative images are shown for controls (A) and 8d LPS exposed animals (B). The C/M ratio decreased after 24h and 8d of LPS exposure compared to controls (C). * $p<0.05$ versus controls

Proliferation and apoptosis in the fetal thymus

Cellular apoptosis and proliferation in the fetal thymus were assessed by immunohistochemical analysis for cleaved caspase-3 and Ki67 in the thymic cortex. The number of cleaved caspase-3 positive cells significantly increased 12h after LPS exposure (27±2 positive cells) compared to controls (12±2 positive cells) (Figure 2A) indicating increased apoptosis in the fetal thymus. Representative images are shown for controls (Figure 2B) and 12h LPS exposed animals (Figure 2C). The percentage of Ki67-positive cells was decreased 4d after exposure to LPS (0.2±0.1%) compared to controls (4.1±1%) indicating a decrease in proliferation (Figure 2D). Representative images are shown for controls (Figure 2E) and 4d LPS exposed animals (Figure 2F).

Accumulation of CD3-positive thymic T-cells

Representative images for CD3 stained thymus tissue are shown for controls (Figure 3A), 5h LPS exposed animals (Figure 3B) and 8d LPS exposed animals (Figure 3C). The percentages of CD3-positive cells in the medulla area of the thymus were significantly increased 5h (32.48±9.43%), 2d (26.5±4.61%) and 8d (26.10±3.60%) after LPS exposure compared to controls (4.22±1.04%)(Figure 3D).

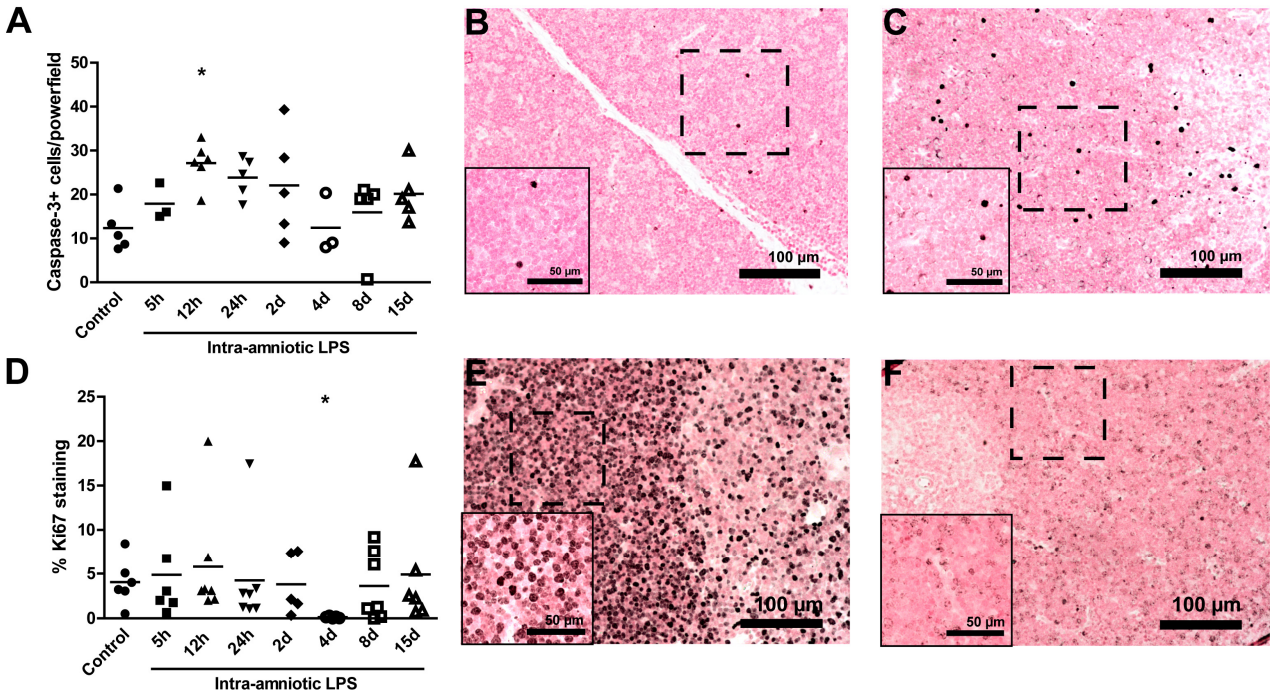

Figure 2: Ki67 and cleaved caspase-3 expression in the fetal thymus. The number of cleaved caspase-3 expressing cells increased in the cortex area of the thymus 12h after the exposure to LPS compared to controls (A). Representative images for cleaved caspase-3 expression in the thymus are shown for controls (B) and 12h LPS exposed animals (C). The percentage of Ki67-positive cells decreased in the cortex area of the thymus 4d after the exposure to LPS compared to controls (D). Representative images for Ki67 expression in the thymus are shown for controls (E) and 4d LPS exposed animals (F). * $p<0.05$ versus controls

Reduced Foxp3-positive cells in response to LPS

Foxp3-positive cells were primarily detected in the inner medulla area of the thymus and are shown for controls (Figure 4A), 4d LPS exposed animals (Figure 4B) and 15d LPS exposed animals (Figure 4C). These cells significantly decreased 4d after LPS exposure (2±1 positive cells) when compared to controls (21±4 positive cells) (Figure 4D). After normalization at 8d, Foxp3-positive cells decreased 15d after exposure to LPS (6±3 positive cells).

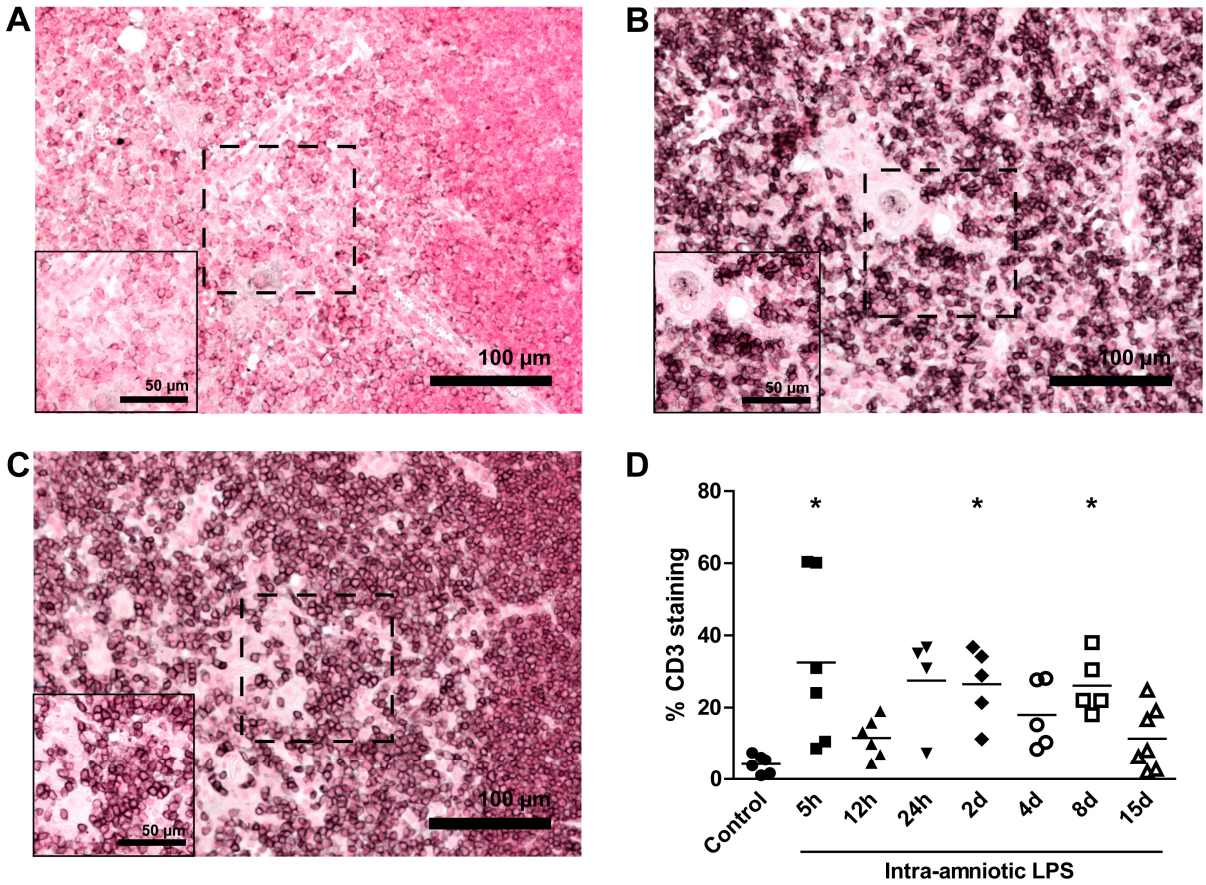

Figure 3: Increased CD3-positive cells in the thymus. The percentage of CD3-positive cells in the medulla area of the thymus was evaluated by immunohistochemistry. Representative images are shown for controls (A), 5h LPS exposure (B) and 8d LPS exposure (C). The percentage of CD3-positive cells was increased in the medulla area of the thymus 5h, 2 and 8d after LPS exposure (D). * $p<0.05$ versus controls

Thymic cytokine mRNA levels in response to intra-amniotic LPS

Interleukin (IL) 1 mRNA levels increased within 24h after LPS exposure and remained elevated until 15d post LPS treatment, with the highest increase of 10-fold detected 4d after LPS treatment (Figure 5A). *IL6* mRNA levels were increased 9-fold at 12h, 24h, 2d and 15d after intra-amniotic LPS exposure (Figure 5B). This *IL6* increase was paralleled by increased *IL17* mRNA levels at 12h, 24h, 4d and 8d post LPS exposure (Figure 5C). A 4-fold increase of *interferon gamma* (*IFNγ*) mRNA levels was detected at 4, 8 and 15d (Figure 5D) whereas *IL10* mRNA levels were significantly decreased by half-fold in the 5h and 2d LPS groups (Figure 5E).

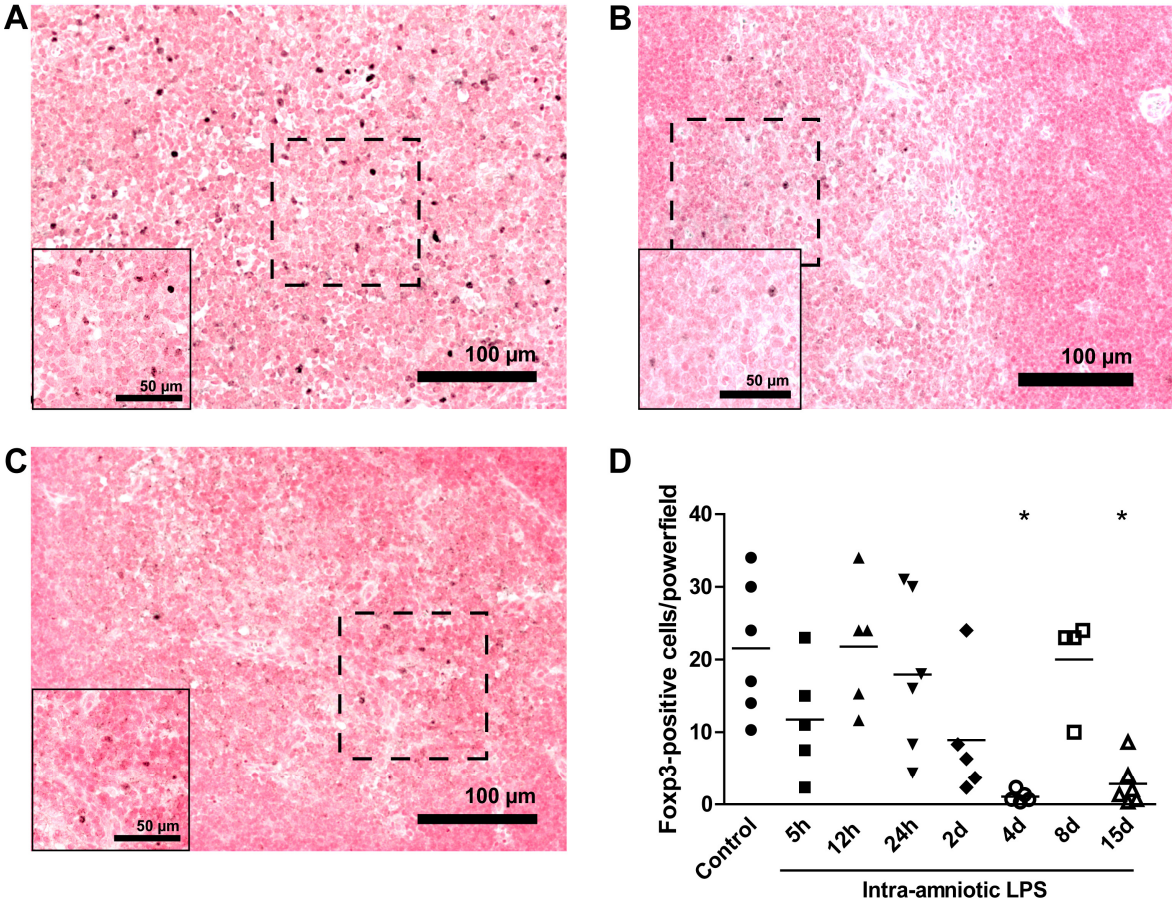

Figure 4: Decreased Foxp3-positive cells after LPS exposure. Representative images for Foxp3 expression in the thymus are shown for controls (**A**), 4d LPS exposure (**B**), and 15d LPS exposed animals (**C**). The number of Foxp3-positive cells decreased in the medulla area 4 and 15d after LPS exposure compared to controls (**D**). * $p<0.05$ versus controls.

Toll like receptor (TLR) mRNA levels in the fetal thymus

The mRNA levels of *Toll-like receptors (TLR) 2* (Figure 6A) and *TLR4* (Figure 6B) in the thymus were analyzed by RT-PCR. *TLR2* mRNA levels were increased at 12h after LPS exposure when compared to controls. The mRNA levels of *TLR4* almost doubled 24h after LPS exposure but decreased to control levels after 2d of LPS exposure. A second significant increase in *TLR4* mRNA levels in the fetal thymus was detected 8d and 15d after LPS exposure.

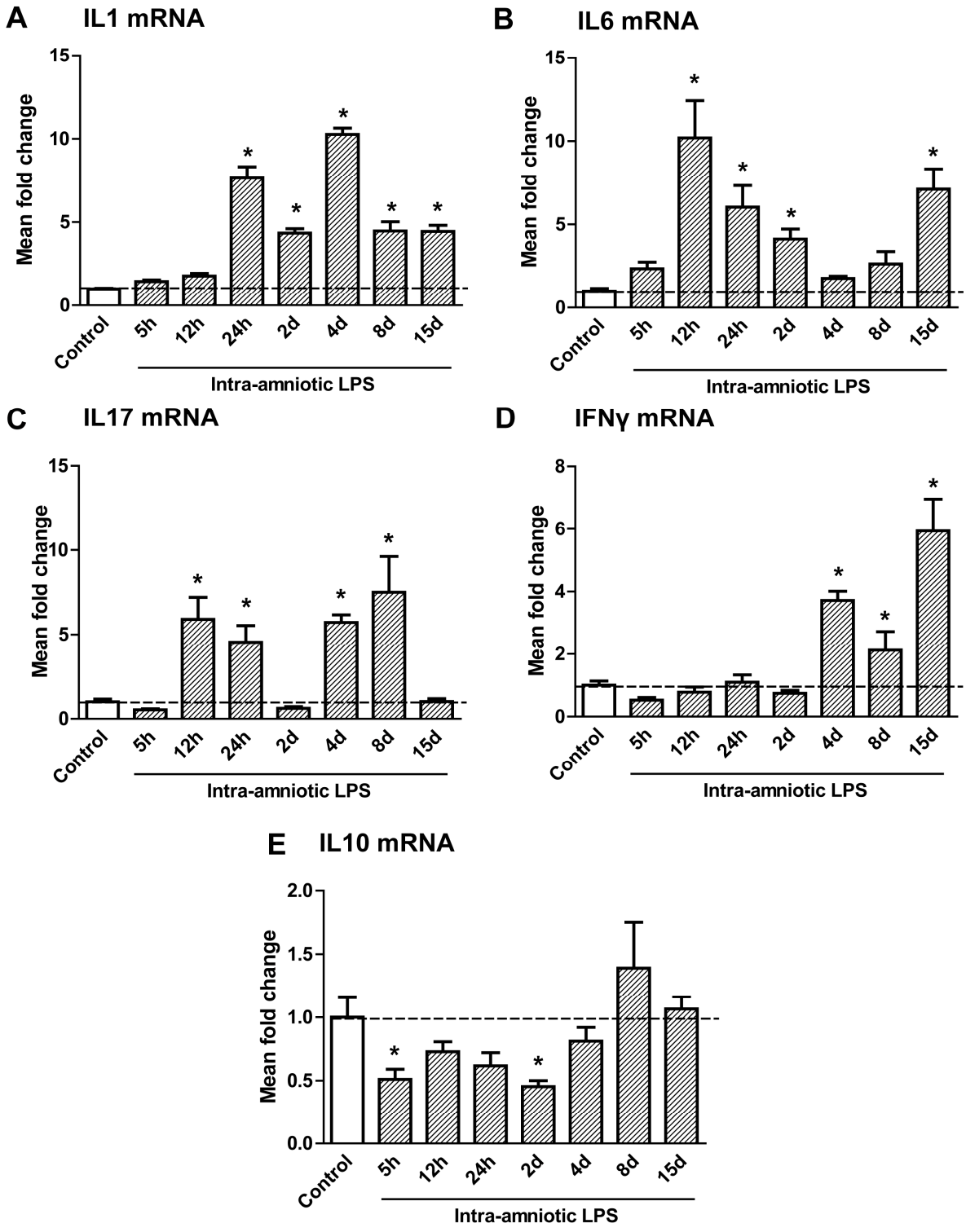

Figure 5: Cytokine mRNA profile. *IL1* mRNA levels (A) increased from 24h up to 15d after the exposure to LPS. mRNA levels of *IL6* (B) increased significantly 12, 24h, 2 and 15d after the LPS exposure compared to controls. *IL17* mRNA levels (C) were increased 6-fold at time points 12, 24h, 4 and 8d. *IFNγ* mRNA (D) increased up to 5-fold 4, 8 and 15d after LPS exposure whereas *IL10* mRNA levels decreased 5h and 2d after the exposure. * $p<0.05$ versus controls.

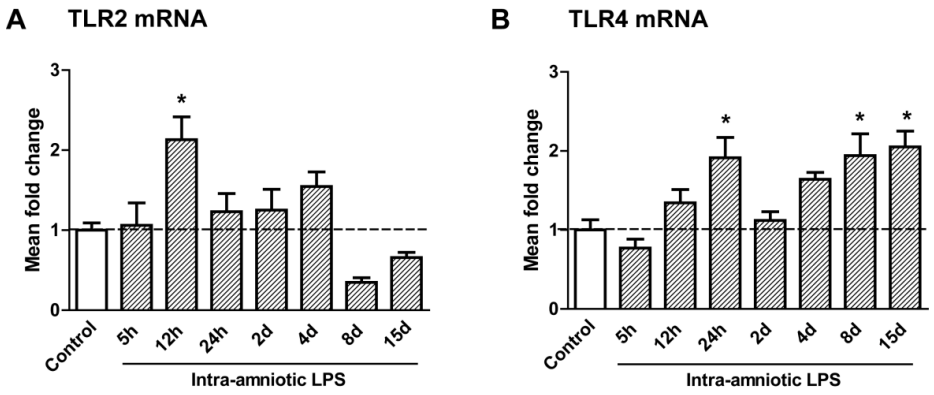

Figure 6: mRNA levels of Toll-Like Receptors (TLR) 2 and 4 in the fetal thymus. *TLR2* (A) mRNA increased 12h after LPS exposure compared to controls. *TLR4* (B) mRNA was increased 2h, 8 and 15d after LPS exposure in the fetal thymus. * p<0.05 versus controls.

Increased numbers of NF-κB positive cells after LPS exposure

NF-κB expression was visualized in thymus sections with immunohistochemistry, as shown for controls (Figure 7A) and 8d LPS exposed animals (Figure 7B). Exposure to intra-amniotic LPS increased the number of NF-κB positive cells in the medulla area of the thymus from 3±1 positive cells in control animals to 33±3 positive cells in the animals exposed to LPS 24h before delivery (Figure 7C). NF-κB positive cells increased again to 28±3 positive cells at 8d.

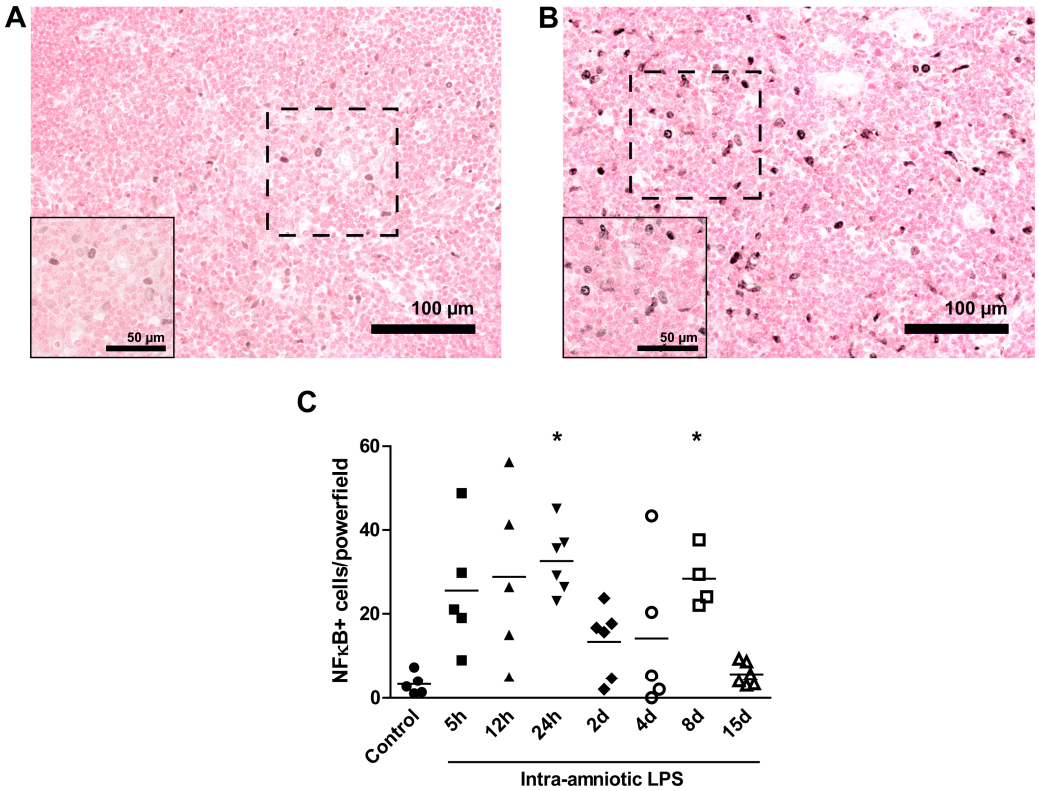

Figure 7: Increased numbers of NF-κB positive cells after LPS exposure. Representative images for NF-κB expression in the thymus are shown for controls (**A**) and 8d LPS exposed animals (**B**). The number of NF-κB positive cells in the medulla area of the thymus increased 24h and 8d after the exposure to intra-amniotic LPS (**C**). * p<0.05 versus controls.

Discussion

We investigated the time interval effects of intra-amniotic LPS exposure on the fetal thymus. In preterm fetal sheep, we found that intra-amniotic exposure to LPS induced a complex response of time dependent acute activation and involution of the fetal thymus, with persistent changes of the thymic T-cell composition and cytokine profiles.

Thymic responses to intra-amniotic LPS exposure were detected as early as 5 hours after the injection. Acute thymic involution was evident with a decrease in the percentage of blood lymphocytes and C/M ratio without large changes in the proliferation or apoptosis rate of the thymocytes. These results of acute thymic involution further refine the findings of Kunzmann et al. which showed an early reduction in thymus/body weight of the ovine thymus 2d after intra-amniotic LPS exposure(33). We found significant apoptosis at 12h and a decreased cortico-medullary ratio at 24h after exposure. We further identified early increases in mature, CD3-positive T-cells and a shift in mRNA cytokine and TLR levels to a pro-inflammatory micro-environment which is supported by an increase in NF-κB expression. TLR4 is the main recognition receptor for LPS. Binding of LPS to TLR4 results in induction of the MyD88-dependent pathway which involves the early phase of NF-kB activation and subsequent production of pro-inflammatory cytokines which in turn can further stimulate NF-kB activation. In addition, TLR4 activates the MyD88-independent pathway which is known to result in the late phase of NF-κB signaling(2).

Intra-uterine inflammation can have both acute and long term effect on thymic T-cell composition(29, 36, 39). Melville et al. showed that intra-amniotic LPS exposure decreased fetal thymic CD8+ and MHC class II cells at 7d after intra-amniotic injection in sheep(39). When sheep were exposed repetitively to intra-amniotic LPS at 90, 100 and 110d of gestation, thymic CD4+ cells increased at term and CD8+ cells decreased in the thymus of animals at 7 weeks of age(36). In the current study, changes in both the cytokine profile and cell composition of the thymus were detectable up to 15d after LPS exposure. As cytokines play a major role in directing thymic T-cell composition(45), persistent changes in the cytokine profile may explain the altered T-cell subsets in the thymus after intra-amniotic LPS exposure. Recently, our group reported that exposure to intra-amniotic LPS 7d before preterm birth altered thymic Sonic Hedgehog (Shh) and Bone morphogenetic protein (BMP)4 expression(34). The Shh and BMP4 pathways are crucial to the development of immature thymocytes to mature T-cells(20, 43). Cytokines such as IL10 can modulate the expression of Shh and BMP4 during this process. Conversely, Shh signaling can regulate the thymic cytokine profile, demonstrating a close interplay between morphogenesis and cytokines on one hand and thymic T-cell development on the other(18, 46, 48). Taken together, these data demonstrate that intra-amniotic exposure to LPS alters the thymic micro-environment, which should have profound effects on thymic T-cell development and subsequent immune function.

In this model, Foxp3 expression, which is a marker of immunosuppressive regulatory T-cells (22), decreased in response to intra-amniotic LPS exposure. As the thymus is the major site for regulatory T-cell development(6), depletion of these cells can cause an imbalance of the immune system towards a persistent pro-inflammatory status. Long term depletion of Foxp3-positive cells may therefore contribute to the inflammatory responses after chorioamnionitis(26, 30, 35, 49). Wolfs et al. recently showed in the fetal gut that IL-1 induced intra-uterine inflammation caused depletion of Foxp3-positive cells and an increase of CD4-expressing cells, indicating an imbalance between effector and regulatory T-cells(51). Under the influence of pro-inflammatory stimuli such as IL6, regulatory T-cells can lose their Foxp3 expression and convert to Th17 cells, which produce IL17 as a potent pro-inflammatory cytokine(3, 5, 28). Since thymic *IL6* and *IL17* mRNA levels increased after intra-amniotic LPS exposure, it is tempting to speculate that an IL-6-driven phenotype change of regulatory T-cells into Th17 cells might be an important process for the observed depletion of regulatory T-cells in the thymus.

In summary, intra-amniotic LPS induced acute thymic involution, structural changes and inflammation of the thymus. Although most changes were reversible, exposure to LPS induced a prolonged increase in pro-inflammatory cytokines and a depletion of Foxp3-positive cells in the fetal thymus, which may alter the response of the fetal immune system in other organ systems such as the lung, gut and brain. Although the long-term effects of the changes in cell composition and activation of the fetal thymus remain to be further determined, this report highlights the dynamics of the fetal immune response to intra-uterine inflammation. These acute changes contribute to a better understanding of how fetal immune regulation could contribute to postnatal diseases as suggested by clinical studies of surviving preterm babies.

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CHAPTER 6

Ovine fetal thymus response to lipopolysaccharide-induced chorioamnionitis and antenatal corticosteroids

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Abstract

Rationale Chorioamnionitis is associated with preterm delivery and involution of the fetal thymus. Women at risk of preterm delivery receive antenatal corticosteroids which accelerate fetal lung maturation and improve neonatal outcome. However, the effects of antenatal corticosteroids on the fetal thymus in the settings of chorioamnionitis are largely unknown. We hypothesized that intra-amniotic exposure to lipopolysaccharide (LPS) causes involution of the fetal thymus resulting in persistent effects on thymic structure and cell populations. We also hypothesized that antenatal corticosteroids may modulate the effects of LPS on thymic development.

Methods Time-mated ewes with singleton fetuses received an intra-amniotic injection of LPS 7 or 14 days before preterm delivery at 120 days gestational age (term=150 days). LPS and corticosteroid treatment groups received intra-amniotic LPS either preceding or following maternal intra-muscular betamethasone. Gestation matched controls received intra-amniotic and maternal intra-muscular saline. The fetal intra-thoracic thymus was evaluated.

Results Intra-amniotic LPS decreased the cortico-medullary (C/M) ratio of the thymus and increased *Toll-like receptor (TLR) 4* mRNA and CD3 expression indicating involution and activation of the fetal thymus. Increased *TLR4* and CD3 expression persisted for 14 days but Foxp3 expression decreased suggesting a change in regulatory T-cells. *Sonic hedgehog* and *bone morphogenetic protein 4* mRNA, which are negative regulators of T-cell development, decreased in response to intra-amniotic LPS. Betamethasone treatment before LPS exposure attenuated some of the LPS-induced thymic responses but increased cleaved caspase-3 expression and decreased the C/M ratio. Betamethasone treatment after LPS exposure did not prevent the LPS-induced thymic changes.

Conclusion Intra-amniotic exposure to LPS activated the fetal thymus which was accompanied by structural changes. Treatment with antenatal corticosteroids before LPS partially attenuated the LPS-induced effects but increased apoptosis in the fetal thymus. Corticosteroid administration after the inflammatory stimulus did not inhibit the LPS effects on the fetal thymus.

Introduction

Preterm birth is the leading cause of morbidity and mortality in the neonatal period (15). In the developed world, the majority of women at risk of preterm birth receive antenatal corticosteroids to induce lung maturation and decrease infant mortality (6). This therapy is given irrespective of the presence of an intra-uterine infection of the amniotic fluid and placental membranes (chorioamnionitis). Chorioamnionitis is present in up to 60% of preterm births and is highly associated with adverse neonatal outcomes (16). In the majority of preterm births, chorioamnionitis is clinically silent prior to early gestational preterm labor (16). As a result, many preterm infants are exposed to both chorioamnionitis and antenatal corticosteroids. Exposure to intra-uterine infection may increase the risk for respiratory and neurological complications in later life (21, 43). Intra-amniotic lipopolysaccharide (LPS)-induced chorioamnionitis causes lung (23, 33), gut (49) and skin (25) inflammation in preterm lambs, which demonstrates that chorioamnionitis causes a `multi-organ disease of the fetus` (13). The concept of fetal and early life origins of disease has developed from epidemiological studies, which correlate fetal and maternal exposures during gestation to outcomes in childhood such as asthma (14). The pathogenesis of some diseases may result from altered T-cell immunity during fetal development (41). The net outcome of pro-inflammation effects from chorioamnionitis and anti-inflammation effects from antenatal corticosteroids remain unstudied. As such, there is minimal information about how the fetal thymus responds to these clinically relevant exposures (28).

The thymus is the primary site for T-cell development (39). Immature T-cells migrate from the cortico-medullary junction, move through the thymic cortex to the medullary compartment. During this migration, the immature T-cells proliferate greatly, alter antigen expression and rearrange their T-cell receptor expression (39). Previous studies demonstrated that chorioamnionitis interferes with the development of the fetal thymus (12, 51). In an ovine model of chorioamnionitis, Kunzmann *et al.* (32) showed that intra-amniotic LPS decreased the fetal thymus/body weight ratio and decreased thymic Foxp3 expression. However, the combined effects of chorioamnionitis and antenatal corticosteroids on fetal thymic development remain to be characterized (29).

Sonic hedgehog (Shh) and Bone morphogenetic protein (BMP) pathways participate in T-cell development and are sensitive to prenatal events such as exposure to toxins (9, 20, 35). Both morphogens are produced and secreted by the thymic epithelium as negative regulators of T-cell differentiation to maintain a pool of undifferentiated, precursor T-cells in the thymus (19, 42). We hypothesized that intra-amniotic exposure to LPS causes involution of the fetal thymus and modulation of Shh and BMP4 expression with persistent effects on thymic structure and cell populations. We also hypothesized that antenatal corticosteroids may modulate the effects of LPS on thymic development. Therefore, we exposed fetal sheep sequentially to intra-amniotic LPS and/or antenatal corticosteroids at 7-day intervals (30) and evaluated multiple indicators of thymic development. An interval of 7 days between the

two interventions was chosen as representative of the interval between recognition of preterm labor and delivery for many women who deliver preterm and the probability that many early gestation fetal exposures to infection are chronic (10, 16), and that repeated administration of antenatal corticosteroids are given at weekly intervals (5).

Materials and methods

Animal study

The animal experiments for this study were performed in Western Australia and were approved by the Animal Ethics Committees at The University of Western Australia (animal ethics protocol RA/3/100/830) and Cincinnati Children`s Hospital Medical Center. Time-mated ewes with singleton fetuses were randomly allocated to one of six treatment groups to receive an intra-amniotic (IA) injection of lipopolysaccharide (LPS) (10 mg Escherichia Coli 055:B5, Sigma Chemical, St. Louis, MO, USA) and/or an intra-muscular injection of betamethasone (Beta) (Celestone Soluspan, Schering-Plough, North Ryde, New South Wales (NSW), Australia, 0.5 mg/kg maternal weight) and/or an equivalent injection of saline for control animals at 107 days and/or 114 days gestation (GA) (Figure 1).

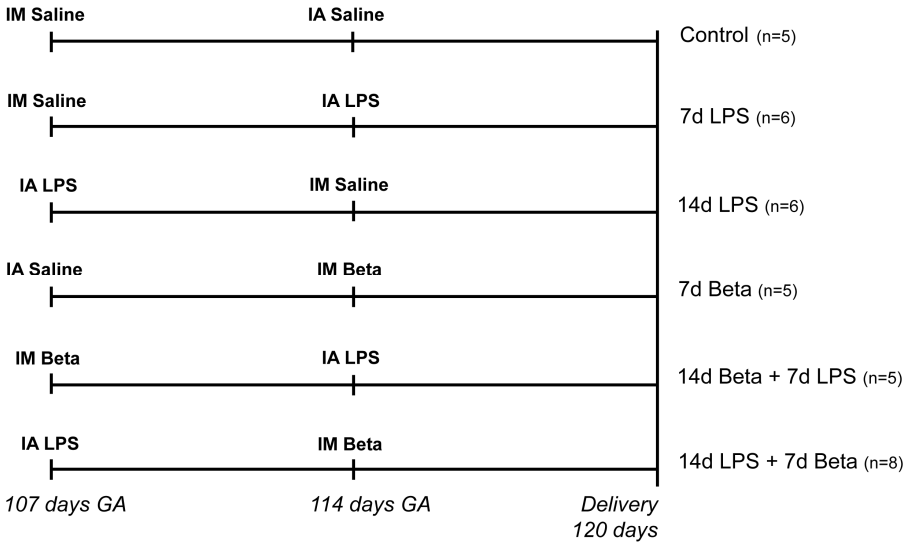

Figure 1: Study design. Pregnant ewes received an intra-amniotic injection of lipopolysaccharide (LPS) and/or a maternal intra-muscular injection of betamethasone (Beta) and/or an equivalent injection of saline for control animals at 107 days and/or 114 days gestation (GA). Lambs were delivered by cesarean section at 120 days GA (term = 150 days GA).

All ewes received a single intra-muscular injection of 150 mg medroxyprogesterone acetate (Depo-Provera, Kenral, NSW, Australia) at 100 days GA to decrease the risk of preterm birth induced by the betamethasone treatment. Despite the medroxyprogesterone acetate treatment, animals exposed to maternal betamethasone experienced fetal losses, such that we reassigned animals from a group which received betamethasone 14 days before delivery to other groups as our priority was to test the interactions of betamethasone and LPS. Lambs were delivered by cesarean section at 120 days GA (term = 150 days GA) and euthanized

after birth. Intra-thoracic thymic tissue was snap frozen and fixed in 10% buffered-formalin for 24 hours. The pulmonary inflammation and maturation responses of these animals are reported elsewhere (33) .

Immunohistochemistry

Paraffin embedded thymic sections (4 µm, transverse) were stained for CD3 (DAKO A0452, DAKO Denmark), Foxp3 (eBiosciences 14-7979, eBiosciences, San Diego, USA), bone morphogenetic protein 4 (BMP4) (sc-6896, Santa Cruz Biotechnology, Santa Cruz, USA), cleaved caspase-3 (Asp175, #9661S, Cell Signaling Technology, Boston, USA) and Ki67 (Dako, M7240, DAKO Denmark). The sections were deparaffinized and rehydrated in an ethanol series. Endogenous peroxidase-activity was blocked by incubation with 0,3% H_2O_2 in phosphate buffered saline (PBS, pH 7.4) (for CD3, BMP4 and Foxp3) or in methanol (for cleaved caspase-3 and Ki67). Antigen retrieval was performed by incubating the sections in heated citrate buffer (10 mM, pH 6.0) for 30 minutes. Aspecific binding was blocked by incubating slides for 30 minutes with 5% bovine serum albumin (BSA) for CD3, 20% normal goat serum (NGS) for Foxp3 and BMP4 or 5% NGS for Ki67. This step was omitted for cleaved caspase-3. Slides were incubated overnight at 4°C with the diluted primary antibody (CD3 1:200, Foxp3 1:30, BMP4 1:500, cleaved caspase-3 1:400, Ki67 1:50) followed by incubation with a secondary goat-anti-mouse (for Foxp3 and Ki67) or swine-anti-rabbit (for CD3, BMP4 and cleaved caspase-3) biotin labeled antibodies. The immunostaining was enhanced with Vectastain ABC peroxidase Elite kit (PK-6200, Vector Laboratories, Burlingame, USA) followed by a nickel sulfate-diaminobenzidine (NiDAB) staining. Sections were counterstained with 0.1% Nuclear Fast Red.

Evaluation was performed by light microscopy (Axioskop 40, Zeiss, Germany) with LeicaQWin Pro v.3.4.0 software (Leica Microsystems, Germany). CD3, Foxp3, Ki67 and BMP4 positive staining were measured in three to five representative sections at 200x magnification by Image J software (Rasband, W.S., Image J US National Institutes of Health, Bethesda, Maryland, USA). Cleaved caspase-3 positive cells were counted in three representative high power fields at 200x magnification by a blinded observer and averaged per animal. The morphology of the thymus was evaluated by light microscopy after hematoxylin and eosin staining. The cortico-medullary (C/M) ratio was quantified for three representative sections from each animal at 2.5x magnification using Image J software (Rasband, W.S.) (47).

RNA extraction and real-time PCR

Total RNA was extracted from frozen thymic tissue using the SV Total RNA Isolation system (Z3100, Promega, Madison, USA) according to the manufacturer`s instructions. Genomic DNA contamination was removed by treatment with RQ1 DNase (M610A, Promega) and the RNA was tested for the presence of genomic GAPDH. Total RNA was reverse transcribed with the First Strand cDNA synthesis kit (4379012001, Roche-Applied, Mannheim, Germany) according to manufacturer`s instructions using anchored oligo-primers. Primers for real-time PCR (RT-PCR) were constructed based on published ovine or bovine cDNA sequences (Table

1). RT-PCR reactions were performed in duplicate with the LightCycler 480 SYBR Green I Master mix (4707516001, Roche-Applied) on a LightCycler 480 Instrument according to the manufacturer`s instructions. RT-PCR results were normalized to ovRSP15, a housekeeping gene, and mean fold changes in mRNA expression were calculated by the ΔΔCt-method (34).

Table 1: Primers used for RT-PCR

Gene		Sequence (5`-3`)	Amplicon size	T^m	Accession code (RefSeq)
TLR2	Fw	GGCTGTAATCAGCGTGTTCA	160bp	64°C	NM_001048231.1
	Rv	GATCTCGTTGTCGGACAGGT			
TLR4	Fw	GAGAAGACTCAGAAAAGCCTTGCT	200bp	65°C	NM_001135930.1
	Rv	GCGGGTTGGTTTCTGCAT			
Shh	Fw	ACTGGAGCGGACCGGCTGAT	82bp	68°C	XM_614193.3
	Rv	CCGGCCACTGGCTCATCAC			
BMP4	Fw	ACCACGAAGAACATCTGGAG	173bp	61°C	NM_001110277.1
	Rv	TTATACGATGAAAGCCCTGC			

Data analysis

Groups were compared using one-way ANOVA with Dunnett's or Tukey's test for post-hoc analysis or by a non-parametric Kruskal-Wallis test as appropriate. Statistical analysis was performed by GraphPad Prism v5.0. Significance was accepted at $p<0.05$.

Results

Thymic cortico-medullary ratio

The cortico-medullary (C/M) ratio decreased significantly after exposure to LPS for 7 days (Figure 2B) compared to controls (Figure 2A). Betamethasone treatment 7 days prior to LPS exposure did not attenuate the change in C/M ratio (Figure 2C). Animals exposed to LPS 14 days before delivery and then exposed to betamethasone had a reduced C/M ratio (Figure 2D) compared to controls.

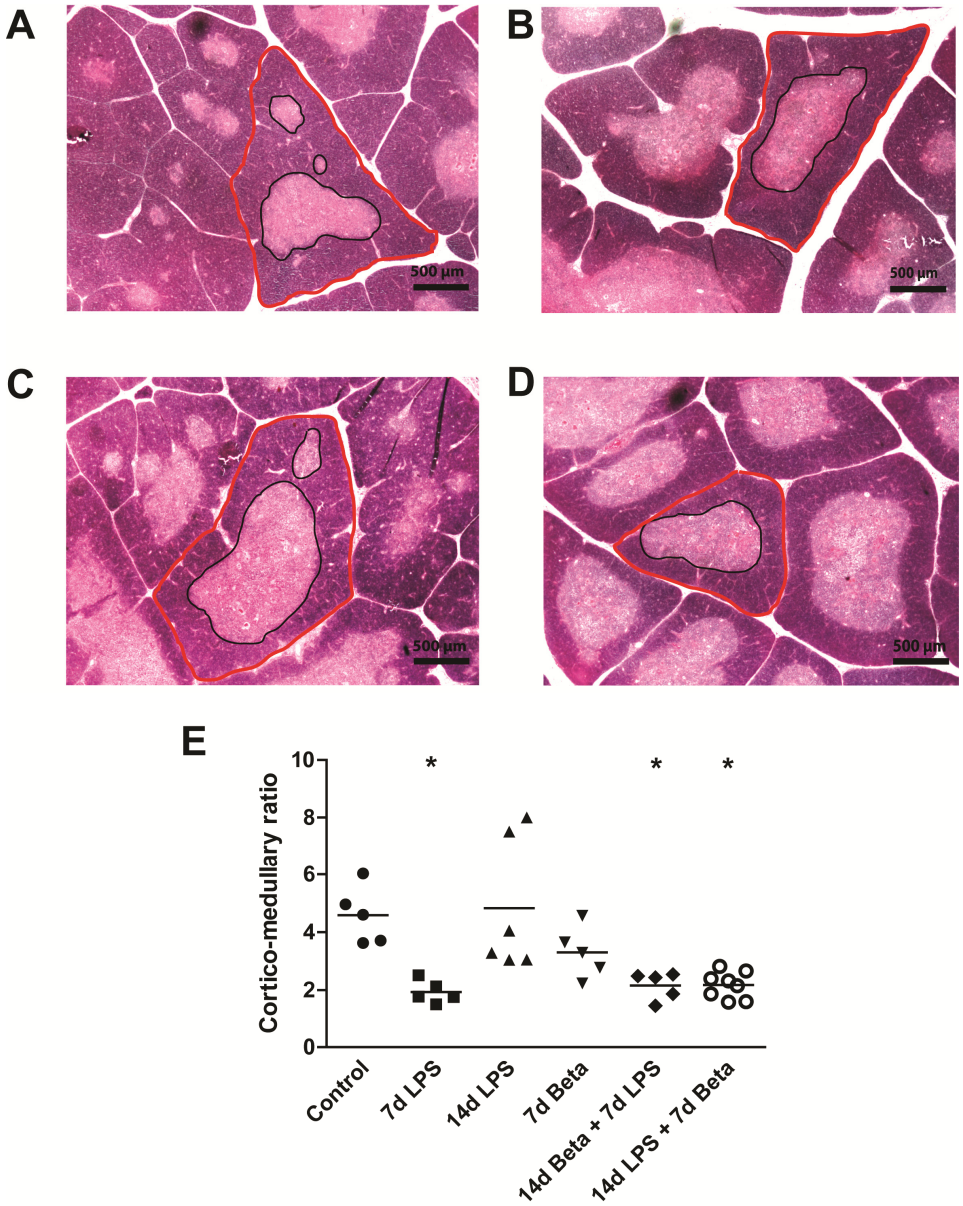

Figure 2: Cortico-medullary ratio. The cortico-medullary (C/M) ratio of the thymus was measured using H&E sections. Representative images are shown for controls (A), 7d LPS (B), 14d betamethasone (Beta) + 7d LPS (C) and 14d LPS + 7d Beta group (D). E: The C/M ratio decreased in the 7d LPS group and the combined LPS and Beta groups. Red circled area: cortex, black circled area: medulla. Magnification: 40x * $p<0.05$ versus controls.

Proliferation and apoptosis

Cleaved caspase-3 positive cells, an indicator of apoptotic cells, increased in the thymus of animals which received betamethasone prior to the LPS exposure when compared to controls (Figure 3A). No changes in Ki67 expression were detected in any of the experimental groups compared to control (Figure 3D).

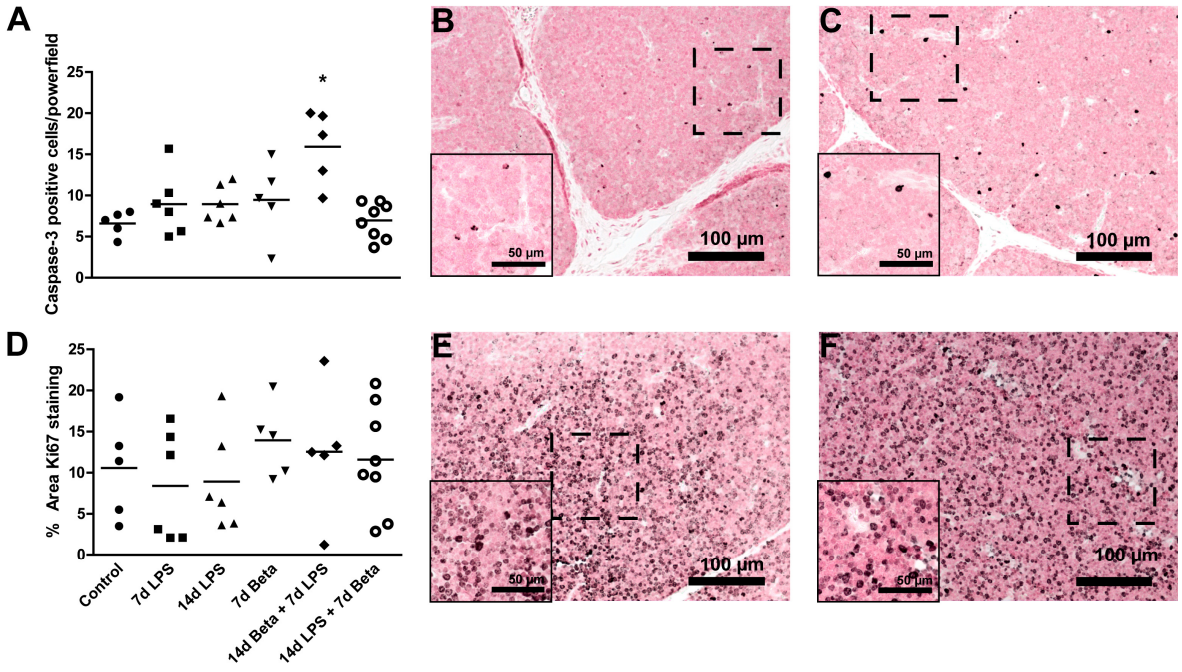

Figure 3: Cleaved caspase-3 and Ki67 expression. A: Cleaved caspase-3 positive cells increased in the thymus in the 14d Beta + 7d LPS group (C) when compared to controls (B). D: No changes in the percentage of Ki67-positive stained area were detected in any of the experimental groups compared to control. Representative images are shown for controls (E) and 7d LPS animals (F). Magnification 200x; magnification insert: 400x.* $p<0.05$ versus controls.

TLR expression in the fetal thymus

TLR2 mRNA levels did not change in the experimental groups compared to control (Figure 4A). *TLR4* mRNA almost doubled in animals which were exposed to LPS either 7 or 14 days before delivery (Figure 4B). Treatment with betamethasone 7 days after the LPS exposure did not attenuate the rise in *TLR4* mRNA levels.

CD-3 positive thymic T-cells

The percentage of CD-3 positive stained area increased significantly 7 days (Figure 5B) and 14 days (Figure 5C) after LPS exposure compared to controls (Figure 5A) and was primarily located in the thymic medulla (Figure 5C). Betamethasone treatment after the 14 day LPS exposure did not attenuate the LPS-mediated increase in the percentage of CD3-positive stained area of the thymus (Figure 5D).

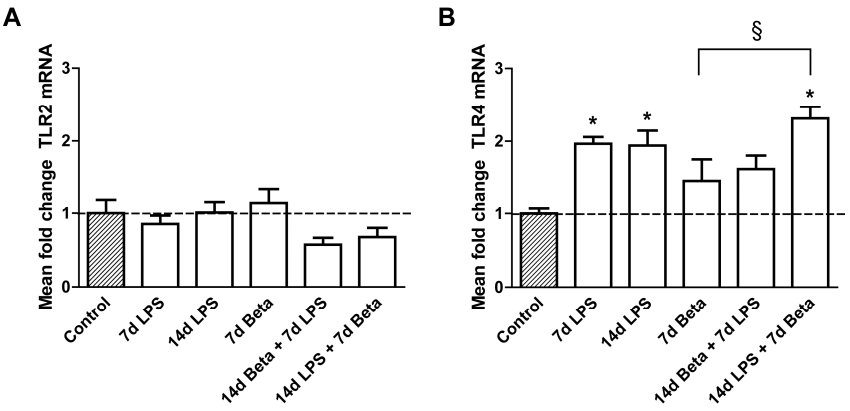

Figure 4: Expression of Toll-Like Receptors (TLR) 2 and 4. *TLR2* (A) was not differently expressed in experimental groups compared to controls. *TLR4* (B) mRNA increased in the 7d LPS, 14d LPS and the 14d LPS + 7d Beta group. * p<0.05 versus controls and § p<0.05 between experimental groups.

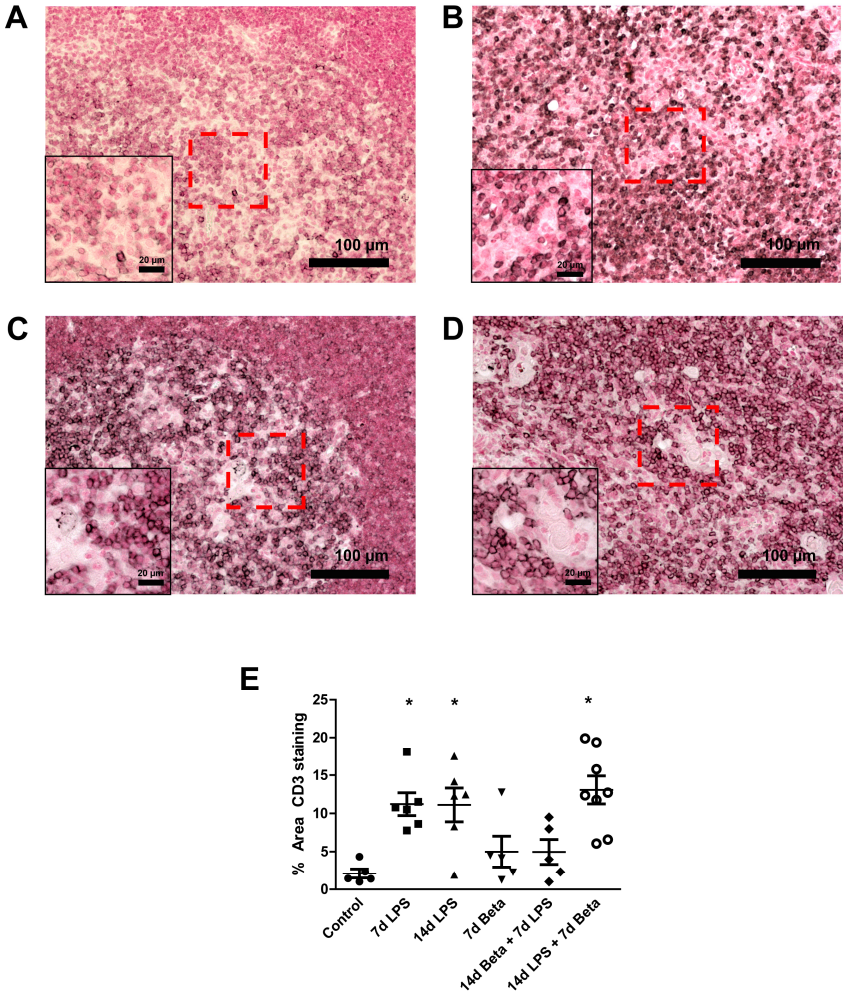

Figure 5: CD3-positive cells in the thymus. The percentage of CD3-positive stained area in the thymus was evaluated by immunohistochemistry. Representative images are shown for controls (A), 7d LPS (B), 14d LPS (C) and 14d LPS + 7d Beta group (D). E: The percentage of CD3-positive area increased in the 7d LPS, 14d LPS and 14d LPS + 7d Beta group. Magnification 200x; magnification insert: 400x. * p<0.05 versus controls.

Decreased Foxp3 expression in response to LPS

The percentage of Foxp3-positive stained area detected primarily in the medulla, was decreased significantly 14 days after LPS exposure (Figure 6B) compared to controls (Figure 6A) irrespectively of betamethasone post-treatment (Figure 6C). Other experimental groups did not show a change in thymic Foxp3 expression.

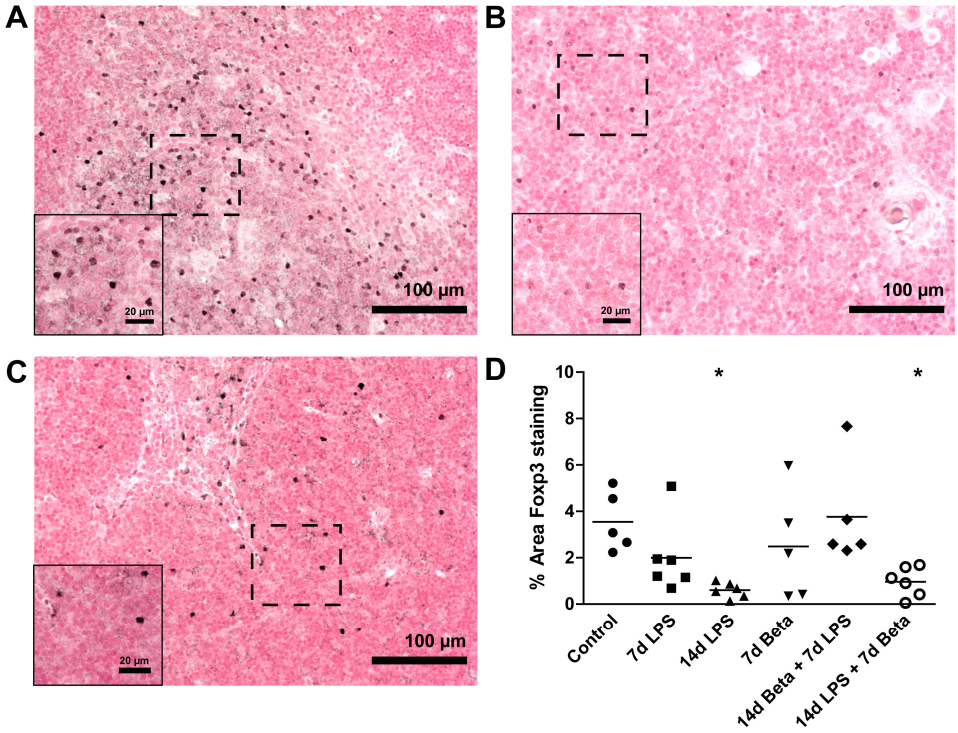

Figure 6: Foxp3-positive cells in the thymus. Representative images for Foxp3 expression in the thymus are shown for controls (A), 14d LPS (B), and 14d LPS + 7d Beta group (C). D: The percentage of Foxp3-positive stained area in the thymic medulla decreased in the animals exposed to 14 days of LPS independent of Beta treatment. Magnification 200x; magnification insert: 400x. * $p<0.05$ versus controls.

Shh and BMP4 expression in the thymus

Shh mRNA (Figure 7) decreased to about 20% of the control value 7 and 14 days after LPS exposure. Similarly, BMP4 (Figure 8) mRNA and protein expression also decreased significantly 7 and 14 days after exposure to LPS. Betamethasone treatment before the exposure to LPS attenuated the decrease in *Shh* mRNA levels and BMP4 protein. *BMP4* mRNA but not protein expression remained decreased in this group compared to controls. The animals which received betamethasone treatment after the LPS exposure still had decreased levels of *Shh* and BMP4 which were similar to the LPS effect alone.

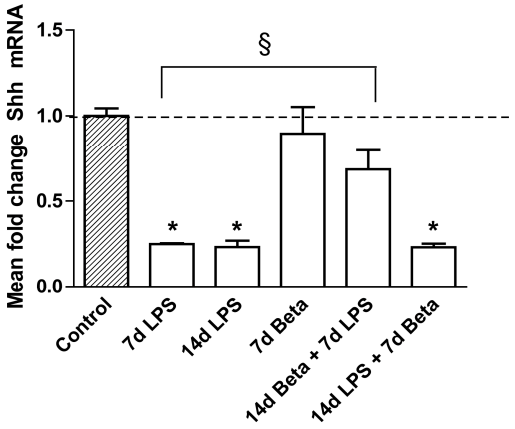

Figure 7: Sonic Hedgehog (Shh) mRNA expression. *Shh* mRNA levels decreased after 7d and 14d LPS and in the 14d LPS + 7d Beta group. * p<0.05 versus controls and § p<0.05 between experimental groups.

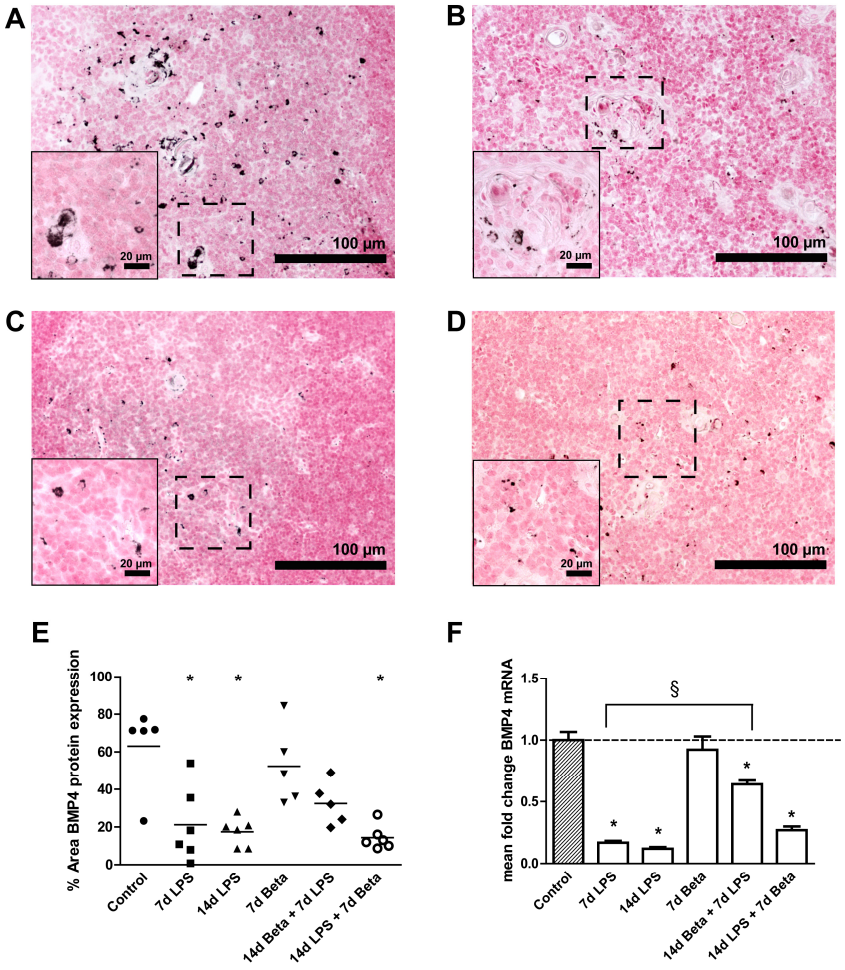

Figure 8: Bone morphogenetic protein 4 (BMP4) expression. Representative images for BMP4 expression are shown for controls (A), 7d LPS (B), 14d LPS (C) and 14d LPS + 7d Beta group (D). E: BMP4 protein expression decreased in the 7d and 14d LPS and the 14d LPS + 7d Beta groups. F: *BMP4* mRNA levels decreased 7 and 14 days after LPS exposure irrespective of betamethasone treatment. * p<0.05 versus controls and § p<0.05 between experimental groups.

Discussion

We investigated the responses of the fetal thymus to chorioamnionitis and antenatal corticosteroids, fetal exposures which are common prior to very preterm delivery (15, 16). We found that intra-amniotic exposure to LPS activated the fetal thymus as shown with increased *TLR4* mRNA levels and CD3 expression, decreased Foxp3-positive cells and altered thymic structure.

In organ cultures of the fetal thymus, blocking of Shh signaling accelerated T-cell differentiation (38) while additional Shh protein arrested T-cell development (18). Cortical epithelial cells of the thymus also produced BMP4 which controls early T-cell development (7). Inhibition of the BMP4 signaling cascade was required for further differentiation of T-cells at several checkpoints during development (17). *Shh* and BMP4 expression decreased in response to LPS-induced chorioamnionitis indicating increased differentiation of thymic T-cells. This increased differentiation was reflected in an increase in CD3 expression, which is expressed on mature T-cells (11). Taken together these results indicate that exposure to intra-amniotic LPS resulted in differentiation of T-cells with an accumulation of mature T-cells in the medulla and depletion of early progenitor T-cells in the cortex, which was consistent with the changed thymic structure.

Although the involution response of the fetal thymus has been described in several human and animal studies (47, 51), the mechanistic changes behind this response remain unclear. Kunzmann et al. (32) showed an acute thymic involution with changes in Foxp3-positive cells in an ovine model of chorioamnionitis up to 5 days after exposure to LPS . Here, we further characterized this process by demonstrating that the effects of LPS on the thymic population and structure were detected 14 days after the LPS exposure and were not due to changes in proliferation or apoptosis. A persistent increase in medulla area due to the accumulation of mature, differentiated T-cells may explain the change in thymic structure.

Based on the anti-inflammatory properties of antenatal corticosteroids, a reduced inflammatory response after exposure to LPS was expected (22). Corticosteroids can exert anti-inflammatory effects by upregulation of the IκB family, which are cytoplasmic inhibitors of NF-κB, and by direct antagonism between the glucocorticoid receptor and NF-κB, resulting in blocked transcription of responsive genes. However, in our study betamethasone administration after the inflammatory stimulus did not reverse the LPS-induced increase in *TLR4* and CD3 in the fetal thymus. LPS has a half-life of 1.7 days in the amniotic fluid and was still detectable 15 days after intra-amniotic injection (37). Because of the slow clearance, LPS may induce a persistent inflammatory response which is in line with measurements of pulmonary inflammation in these animals (33) .

Surprisingly, betamethasone administration 7 days before LPS exposure attenuated activation with no signs of inflammation in the fetal thymus. Thymic structure changed

slightly due to the pro-apoptotic properties of antenatal corticosteroids (46). Previous reports demonstrated only inhibitory effects of corticosteroids on the immune system for a maximum of 48 hours (26, 48). Our results indicate that the antenatal corticosteroids used clinically can potentially desensitize the fetal immune system and attenuate a response to LPS. Paradoxically, these `longer term` inhibitory effects of corticosteroids on the fetal immune system did not occur in the animals that were exposed to LPS and then betamethasone 7 days later as the immune system remained activated. Corticosteroids are potent immune-modulatory hormones which can have long term effects on the HPA-axis and subsequently on the function of the immune system (4, 44) which may be reflected in the unresponsiveness of the fetal immune system to LPS after corticosteroid pre-treatment. The longer-term effects of the changes in cell composition and activation of the fetal thymus after exposure to antenatal corticosteroids may depend on the timing of the exposure and the developmental stage of the immune system and therefore remain to be further determined.

Although administration of antenatal corticosteroids to pregnant women at risk of preterm birth is one of the most effective and important therapies in perinatal medicine, concerns remain about effects on fetal growth and development of the brain and immune system. Antenatal corticosteroid treatment can change the population and function of cord blood lymphocytes of preterm infants (8, 24) and may induce thymic involution (36, 40, 50). Antenatal dexamethasone also was associated with decreased T-cell numbers in the fetal rat thymus and spleen and changes in the CD4/CD8 ratio (3, 4). Dexamethasone treatment of neonatal rats changed the peripheral T-cell repertoire and altered endogenous corticosterone production of thymic epithelial cells during neonatal life (2). These changes may impair the functional maturity of the neonatal immune system and could contribute to the increased incidence and adverse outcome of infections (1, 45).

Our findings contribute to the current concept that events during fetal life can potentially alter the function of the immune system (27). The clinical associations between chorioamnionitis and adverse outcomes in later life such as BPD (41) or asthma (31) may be mediated in part by changes in immune responses. In summary, our results demonstrate that fetal exposure to intra-amniotic LPS activated the fetal thymus which was accompanied by structural changes. Treatment with antenatal corticosteroids before LPS partially attenuated the LPS-induced effects but increased apoptosis in the fetal thymus. Corticosteroid administration after the inflammatory stimulus did not inhibit the LPS effects on the fetal thymus. However, insights into the effects of LPS and corticosteroids on molecular pathways such as BMP4 and Shh are limited. Due to the low expression BMP4 and a lack of specific reagents for Shh protein for ovine tissue, we were not able to perform more detailed analysis of these pathways. Further analysis at different time intervals of exposure are necessary to better understand the interactive effects of chorioamnionitis and corticosteroids on the fetal thymus. Although the design of the study does not allow us to

evaluate the dynamics of the changes induced by LPS and corticosteroids, this report illustrates the complicated interactions of pro- and anti-inflammatory stimuli on the development of the fetal immune system.

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Chapter 7

Splenic responses to intra-amniotic lipopolysaccharide (LPS) exposure in fetal sheep

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Submitted

Abstract

Rationale Intra-uterine exposure to inflammation affects the fetal immune system which can increase the susceptibility for postnatal infections and sepsis in preterm infants. We investigated the splenic cell composition and inflammatory response following *in utero* intra-amniotic lipopolysaccharide (LPS) exposure over time.

Methods Fetal sheep were exposed to intra-amniotic LPS 5, 12 or 24h or, 2, 4, 8 or 15d before delivery at 125d gestational age (term=150d).

Results LPS exposure increased splenic *IL1*, *IL10* and *IFNγ* mRNA levels and RelB expression up to 15d after the onset of intra-uterine inflammation. Cleaved caspase-3 positive cells were increased 2d and 8d after the LPS exposure. Splenic CD3 immuno-reactivity increased as early as 5h after the LPS injection and was accompanied by increased numbers of Foxp3-postive cells at 12h post injection.

Conclusion The fetal spleen mounts a persistent pro-inflammatory response following intra-amniotic LPS exposure which may contribute to immune dysfunction in postnatal life.

Introduction

Exposure to intra-uterine inflammation is a major risk factor for spontaneous preterm birth and adverse neonatal complications (10, 27). Chorioamnionitis, a poly-microbial inflammation of the chorio-amniotic membranes, is present in up to 60% of all preterm births below 25 weeks of gestation (11). Exposure of the fetus to intra-uterine inflammation through contact with the contaminated amniotic fluid or through the placental-fetal circulation can induce a fetal systemic response referred to as the fetal inflammatory response syndrome (FIRS)(12). The presence of FIRS is associated with an increased risk for respiratory (4, 15), neurodevelopmental (2, 5) and intestinal complications (8) in preterm infants leading to postnatal diseases such as bronchopulmonary dysplasia (13), cerebral white matter injury (31) and necrotizing enterocolitis (3).

Preterm infants are highly prone to develop postnatal infections and sepsis (30). This increased susceptibility can be attributed to various pre- (e.g. lack of maternal antibodies) and postnatal factors (e.g. mechanical ventilation). Intra-uterine exposure to inflammation is one of the prenatal events that appear to be associated with the increased incidence of postnatal infections in preterms (1, 32). Clinical studies showed that neonates born after exposure to chorioamnionitis have an altered immune response (1) which increases the risk for further organ dysfunction and early onset sepsis (7, 9, 35). Histological examination of the spleen and thymus of fetuses exposed to chorioamnionitis or neonatal sepsis showed severe leukocyte depletion and morphological changes (33, 34). Although these studies suggest that exposure to inflammation during gestation can have profound effects on the immune system of preterm infants, little information exists about the mechanisms by which intra-uterine inflammation can modulate the fetal immune system, in particular the immunological organs (8, 35). Recently, we and others showed that exposure of fetal lambs to intra-amniotic lipopolysaccharide (LPS)-induced inflammation causing chorioamnionitis (20), resulted in involution of the thymus and persistently altered the thymic micro-environment and T-cell composition which may modulate the response of the immune system *in utero* and later life (14, 17, 18, 21-23, 25). As clinical studies also postulate that intra-uterine inflammation affects the fetal spleen, we characterized the inflammatory response of the spleen over time in a preterm lamb model of LPS-induced chorioamnionitis.

Materials and Methods

Experimental animal study

The experimental animal study was approved by the Animal Ethics Committees at The University of Western Australia and Cincinnati Children`s Hospital Medical Center, Ohio, USA. The design of this study was published previously(23). In brief, time-mated Merino ewes with singleton fetuses were randomly allocated to receive an intra-amniotic injection of 10 mg LPS by ultrasound guidance (*Escherichia Coli* 055:B5, Sigma Chemical, St. Louis,

USA, dissolved in saline) 5 hours (h), 12h, 24h, 2 days (d), 4d, 8d or 15d (n=6) before delivery at 125 days gestational age (GA) (term = 150 days GA) (Figure 1) (35). Control animals (n=6) received an intra-amniotic injection with saline. The preterm lambs were euthanized after surgical delivery and splenic tissue was snap frozen or fixed in 10% buffered-formalin for 24 hours.

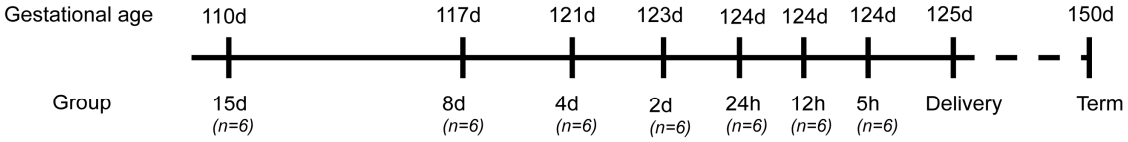

Figure 1: *Study design.* Fetal sheep were exposed to an intra-amniotic LPS injection 5, 12 or 24h or, 2, 4, 8 or 15d before preterm delivery at 125d gestational age (term=150d). Control animals received an intra-amniotic saline injection.

RNA extraction and quantitative real-time PCR

The mRNA levels of *interleukin (IL)1*, *IL6*, *IL10*, and *interferon gamma (IFNγ)* which are cytokines produced in response to inflammation, were measured by quantitative real-time PCR (qPCR) as described previously(23). Total RNA was extracted from snap frozen splenic tissue by Trizol/chloroform extraction. RNA was converted to cDNA with the Transcription First Strand cDNA synthesis kit (Roche-Applied, Mannheim, Germany). qPCR reactions were performed with 5 µl of cDNA in duplicate with the LightCycler 480 SYBR Green I Master mix (Roche-Applied, Mannheim, Germany) on a LightCycler 480 Instrument. Primer sequences are listed in Table 1. qPCR results were normalized to the housekeeping gene ovine 40S ribosomal protein S15 (ovRPS15). Mean fold changes in mRNA expression compared to the control group were calculated by the ΔΔCt-method (24).

Table 1: Primers used for qPCR

Gene		Sequence (5`-3`)	Amplicon size	T^m
IL1	Fw	CACTGCCAGAAAATAAGCTGAAAC	79bp	63°C
	Rv	TGATCAAGCAAATCGCCTGAT		
IL6	Fw	ACATCGTCGACAAAATCTCTGCAA	90bp	65°C
	Rv	GCCAGTGTCTCCTTGCTGTTT		
IL10	Fw	CATGGGCCTGACATCAAGGA	102bp	64°C
	Rv	CGGAGGGTCTTCAGCTTCTC		
IFNγ	Fw	TCAAGCAAGACATGTTTCAGAAGTTCT	65bp	60°C
	Rv	CCGGAATTTGAATCAGCCTTTTGAA		
ovRPS15	Fw	CGAGATGGTGGGCAGCAT	93bp	60°C
	Rv	GCTTGATTTCCACCTGGTTGA		

Immunohistochemistry

Paraffin embedded splenic sections (4 µm, transverse) were immuno-stained for CD3 (marker for mature T-cells, A0452, DAKO, Glostrup, Denmark), Foxp3 (marker for regulatory T-cells, 14-7979, eBiosciences, San Diego USA), cleaved caspase-3 (cellular apoptosis marker,

Asp175, #9661S, Cell Signaling Technology, Boston, USA), Ki67 (proliferation marker, M7240, DAKO, Glostrup, Denmark) and RelB (a component of the NF-κB complex, sc-266, Santa Cruz Biotechnology, Santa Cruz, USA) as reported elsewhere(23).

The sections were evaluated by light microscopy (Axioskop 40, Zeiss, Oberkochen, Germany) with LeicaQWin Pro v.3.4.0 software (Leica Microsystems, Wetzlar, Germany). Five representative images of the stainings were taken at 200x magnification. For CD3 and Ki67, the percentage of positive stained area was measured by applying a standardized threshold using Image J software (Rasband, W.S., Image J US National Institutes of Health, Bethesda, USA). Foxp3 and cleaved caspase-3 positive cells were counted in five representative images at 200x magnification and averaged per animal. Five images of the RelB staining were scored semi-quantitatively according to the following rating: 0 - no staining, 1 - little staining, 2 - medium staining, 3 - heavy staining. All analyses were performed in a blinded manner.

Data analysis

Groups are shown as means ± standard error of mean (SEM). LPS exposed groups were compared to the control group using one-way ANOVA with Dunnett's for post-hoc analysis or by a non-parametric Kruskal-Wallis test as appropriate. Statistical analysis was performed by GraphPad Prism v5.0. Significance was accepted at $p<0.05$.

Results

Splenic cytokine mRNA profile

The mRNA levels of *IL1* increased about two-fold 2d, 8d and 15d after intra-amniotic LPS exposure compared to controls (Figure 2A). Splenic *IL6* (Figure 2B) and *IL1* mRNA levels were decreased by 50% 4d after the LPS injection. *IL10* mRNA increased significantly at 24h, 2d, 8d and 15d post LPS exposure compared to controls (Figure 2C). Splenic *IFNγ* mRNA levels were increased as early as 5h after the LPS injection and were elevated up to 15d after the exposure (Figure 2D).

RelB expression after intra-amniotic LPS exposure

Representative images of splenic RelB expression are shown for controls (Figure 3A) and 2d LPS exposed animals (Figure 3B). The relative RelB expression in the fetal spleen increased significantly 12h, 2d and 15d after intra-amniotic LPS exposure compared to control animals (Figure 3C).

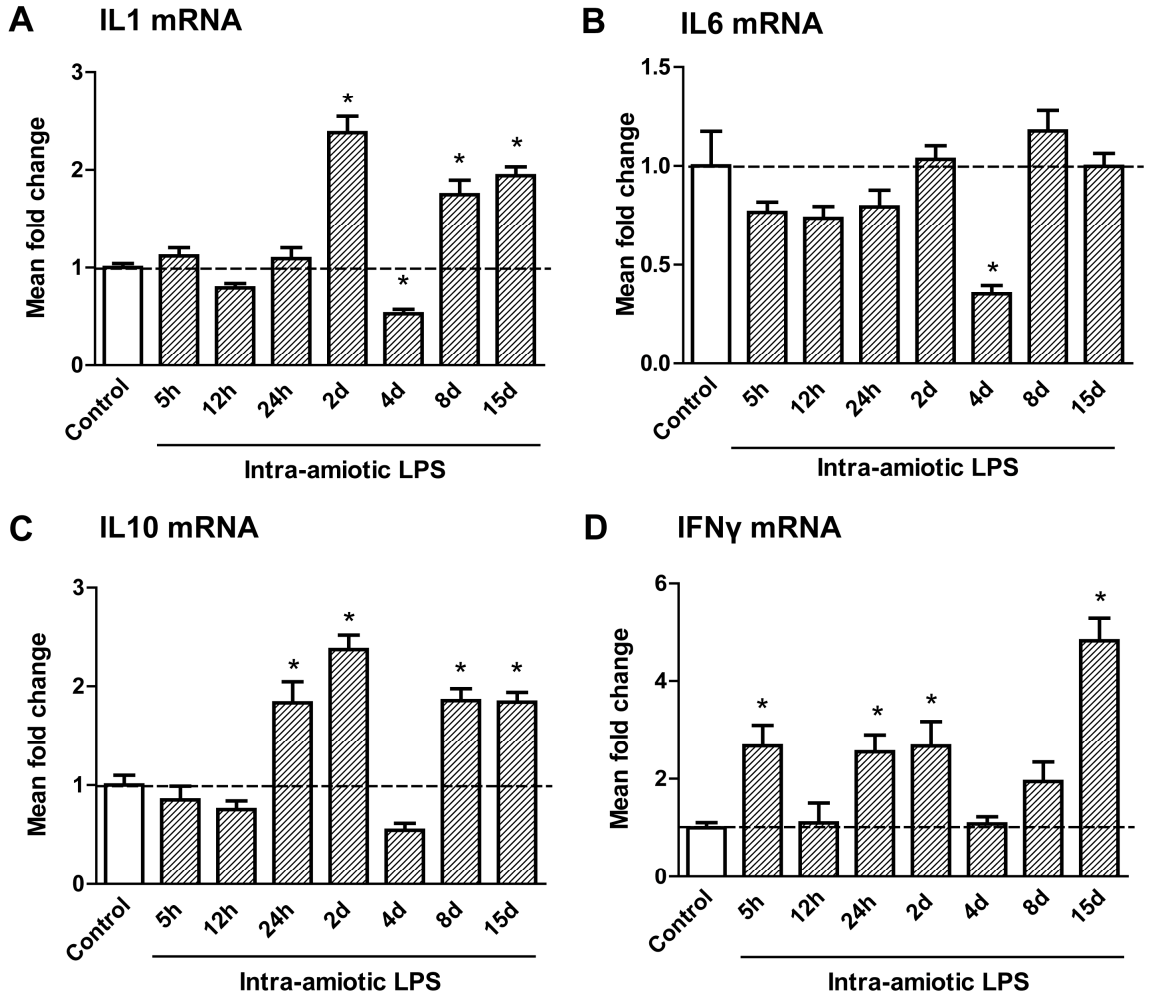

Figure 2: *Splenic cytokine mRNA profile. IL1* mRNA levels (A) increased at 2d, 8d and 15d post LPS exposure and decreased by 50% at 4d post LPS exposure. mRNA levels of *IL6* (B) decreased by 60% 4d after the LPS injection. LPS exposure increased *IL10* mRNA levels (C) 24h, 2d, 8d and 15d after the injection. *IFNγ* mRNA levels (D) were increased 5h, 24h, 2d and 15d after LPS exposure. * $p<0.05$ versus controls.

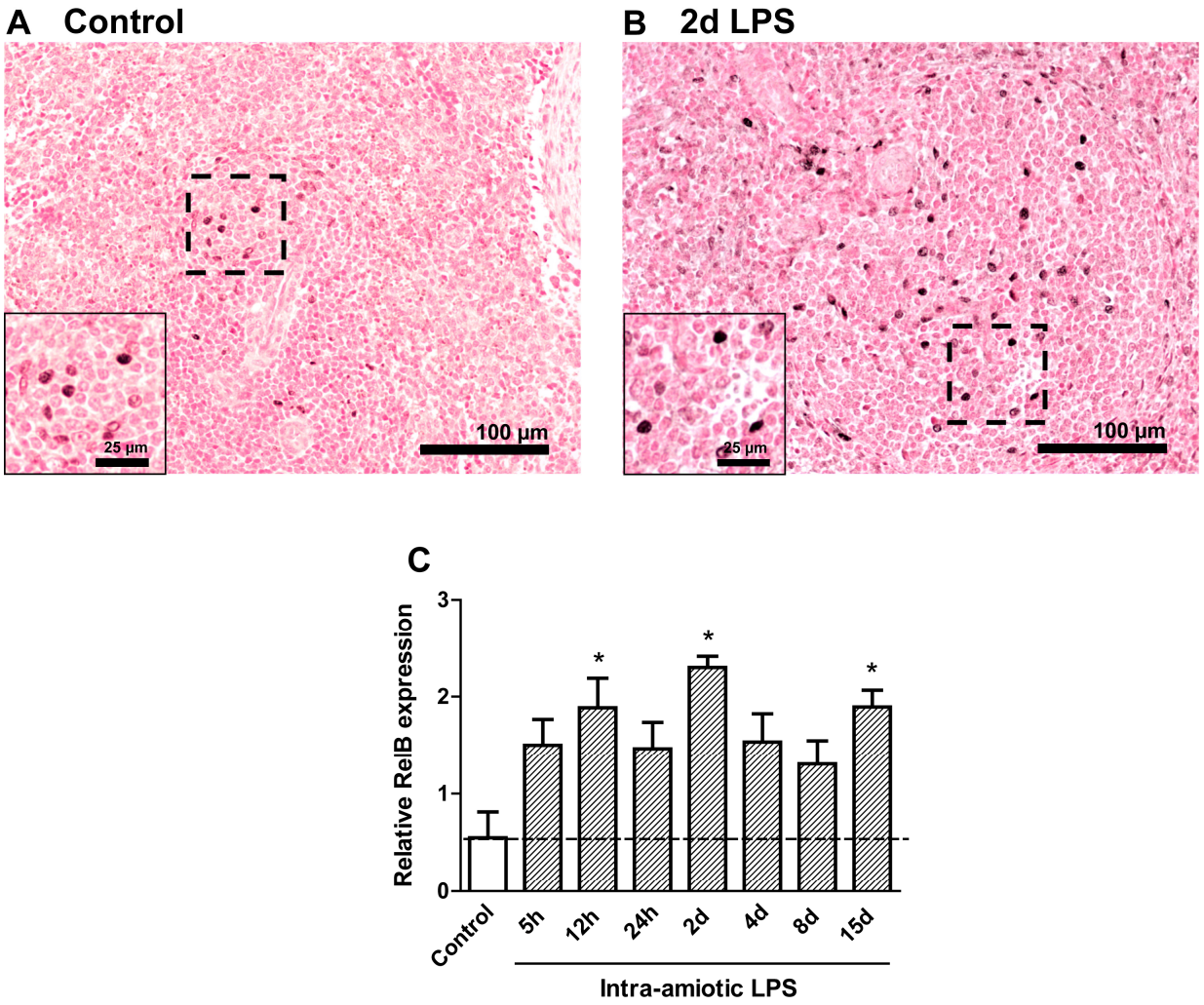

Figure 3: *RelB expression after intra-amniotic LPS exposure.* Representative images for splenic RelB expression are shown for controls (A) and 2d LPS exposed animals (B). The relative RelB expression in the fetal spleen increased in animals exposed to LPS 12h, 2d and 15d before delivery (C). * $p<0.05$ versus controls.

Changes in proliferation and apoptosis in the fetal spleen

Proliferation and apoptosis in the fetal spleen was determined by immunohistochemical staining of Ki67 and cleaved caspase-3 respectively. The number of cleaved caspase-3 positive cells was significantly increased 2d and 8d after the LPS exposure (Figure 4A). Splenic cleaved caspase-3 expression is shown for controls (Figure 4B) and 8d LPS exposed animals (Figure 4C). The percentage of Ki67-positive stained area decreased 15d post LPS exposure compared to control animals (Figure 4D). Representative images for Ki67 expression in the spleen are shown for controls (Figure 4E) and 15d LPS exposed animals (Figure 4F).

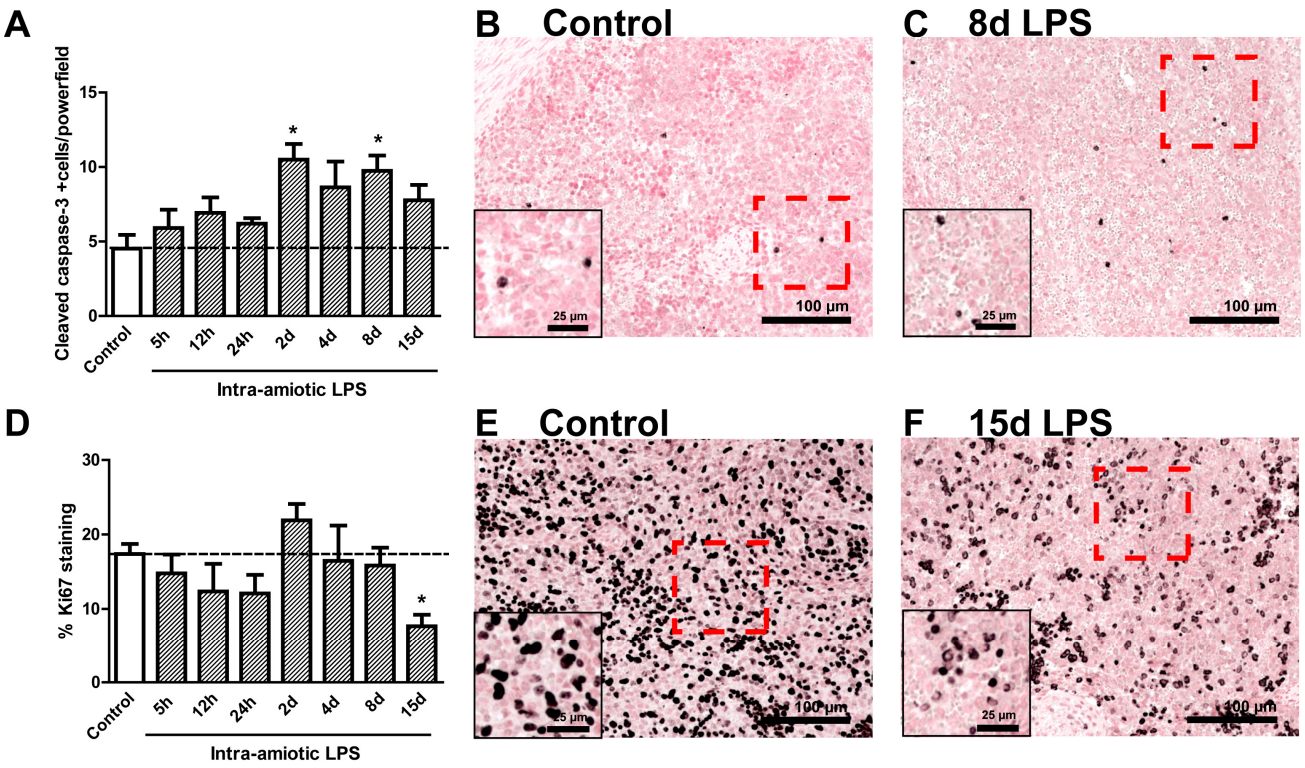

Figure 4: *Cleaved caspase-3 and Ki67 expression in the fetal spleen.* The number of cleaved caspase-3 positive cells in the fetal spleen increased at 2d and 8d after the LPS exposure compared to controls (A). Representative images for cleaved caspase-3 expression are shown for controls (B) and 8d LPS exposed animals (C). The percentage of Ki67-positive stained area in the spleen decreased in animals exposed to LPS 15d before delivery (D). Representative images for Ki67 expression are shown for controls (E) and 15d LPS exposed animals (F). * $p<0.05$ versus controls

CD3-positive T-cells in the fetal spleen

Representative images for the splenic CD3 expression are shown for controls (Figure 5A) and for 5h LPS exposed animals (Figure 5B). Intra-amniotic exposure to LPS increased the percentage of CD3-positive stained area in the fetal spleen as early as 5h after the injection (Figure 5C). CD3 immuno-reactivity increased by two-fold 2d and 4d after LPS exposure compared to controls.

Increased splenic Foxp3-positive cells after LPS exposure

Representative images of Foxp3-positive cells are shown for controls (Figure 6A) and 12h LPS exposed animals (Figure 6B). The number of splenic Foxp3-positive cells increased at 12h after the LPS exposure compared to controls (Figure 6C).

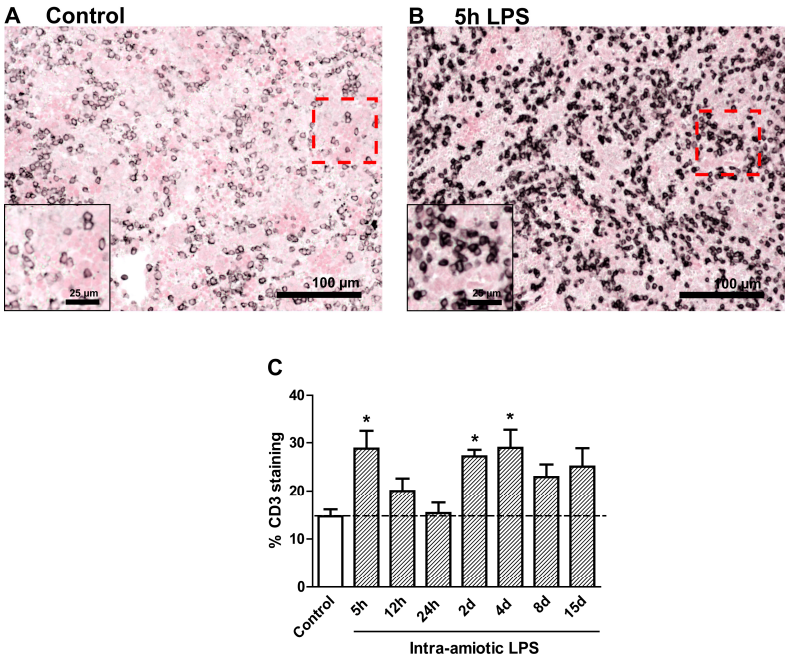

Figure 5: *CD3-positive T-cells in the fetal spleen.* Representative images for CD3 positive staining in the fetal spleen of controls (A) and 5h LPS exposed animals (B). The percentage of CD3-positive stained area increased in animals exposed to intra-amniotic LPS 5h, 2d and 4d before delivery compared to control animals (C). * p<0.05 versus controls

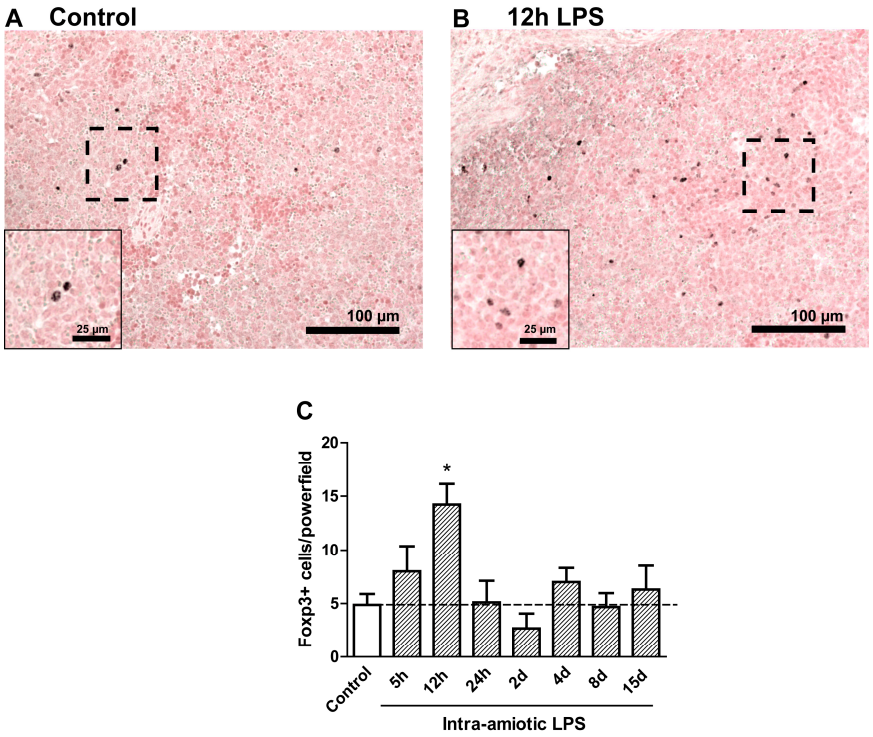

Figure 6: S*plenic Foxp3-positive cells.* Representative images for Foxp3-positive cells in the spleen are shown for controls (A) and 12h LPS exposed animals (B). Intra-amniotic LPS exposure 12h before delivery increased the number of Foxp3-positive cells in the spleen compared to controls (C). * p<0.05 versus controls.

Discussion

We studied the response of the fetal spleen to intra-uterine inflammation over time in a preterm lamb model of chorioamnionitis. We showed that intra-amniotic LPS exposure induced an inflammatory response in the spleen with increased production of cytokines and moderate changes in the cellular composition. These changes were detectable up to 15 days after the onset of inflammation suggesting that exposure to intra-amniotic inflammation can alter the fetal immunological organs over a longer time period.

Although the dynamics of the systemic response to intra-amniotic LPS has been well characterized (14, 20), little is known about the time course of the immunological response of the fetal spleen to LPS. In this study we showed that intra-amniotic injection of 10 mg LPS induced the production of inflammatory cytokines and increased CD3 immuno-reactivity within 5h after administration, with a maximum splenic response 2d after LPS injection. Interestingly, we also detected increased production of *IL10* mRNA, a potent anti-inflammatory cytokine, after intra-amniotic LPS exposure. This suggests that the fetal spleen can locally produce both pro-inflammatory as well as anti-inflammatory cytokines to provide to the systemic circulation which can modify the inflammatory response (29).

Little is known about how the fetus detects an inflammatory stimulus in the amniotic fluid and which organs mount the initial response. Previous studies showed that increased cytokine levels are detected in the fetal lungs as early as 2h after intra-amniotic LPS exposure (14). The fetal skin responds at 12 hours after the injection of an inflammatory stimulus followed by the gut which shows signs of inflammation 3 days after the exposure (36, 37). Here, we demonstrate that the fetal spleen is already capable to mount an inflammatory response following a single dose of 10 mg of intra-amniotic LPS within 5h. The systemic immunological response of the fetus might therefore contribute substantially to the inflammatory process in the fetal gut and skin and might obviate the need for direct contact of these organs to contaminated amniotic fluid in order to induce a local inflammation (16, 19).

Splenic lymphocyte depletion has been described in preterm infants after exposure to severe chorioamnionitis or sepsis (33). In our study, we investigated apoptosis and cell proliferation as a mechanism of possible splenic depletion or involution. We did not detect any major changes in the rate of apoptosis or proliferation neither a decrease in CD3-positive T-lymphocytes in the fetal spleen following intra-amniotic LPS injection. This suggests that exposure to moderate intra-uterine inflammation, as is the case for most surviving preterm infants born after chorioamnionitis, does not induce severe splenic depletion.

Splenic responses to intra-amniotic LPS exposure in this model were detectable up to 15 days after the injection. Cytokine levels of *IL1*, *IL10* and *IFNγ* remained elevated together with an increased expression of the NF-κB protein RelB. We described a similar persistent

pro-inflammatory status after intra-uterine inflammation in the thymus of these animals suggesting long term alterations of the fetal immunological organs after exposure to *in utero* inflammation (22, 23). Dysregulation of the fetal immune system can play a major role in the etiology of other neonatal complications such as bronchopulmonary dysplasia and necrotizing enterocolitis (26, 28). Furthermore, the described changes in the fetal immunological organs may modulate the immune response of the fetus in postnatal life thereby providing a possible mechanism for the clinical association between the presence of chorioamnionitis and the increased incidence of early onset sepsis in preterm infants (1, 6, 9).

In summary, this is the first report to show the response of the fetal spleen following intra-amniotic inflammation in preterm sheep. We demonstrated that intra-amniotic LPS exposure increased splenic cytokine production and NF-κB expression up to 15 days after the injection. This altered immune status of the fetal spleen can contribute to the persistent inflammation and injury response in the other fetal organs and the increased susceptibility of preterms for postnatal infections. There are limitations to this study. We were not able to perform detailed assessments of the cellular or functional changes in the fetal splenocyte sub-populations. Furthermore, the consequences of these acute changes on the long term function of the immune system remain to be elucidated. Despite the acknowledged limitations, this report contributes to a better understanding of how intra-amniotic exposure to inflammation can potentially alter immune regulation in postnatal life.

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CHAPTER 8

Modulation of intra-amniotic lipopolysaccharide-mediated brain inflammation by antenatal glucocorticoids is timing dependent

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Abstract

Rationale Chorioamnionitis is associated with preterm birth and brain injury, both contributing to cognitive and motor deficits in preterm infants. Antenatal glucocorticoids are administered to mothers at risk of preterm delivery to improve neonatal outcomes. However, the effects of antenatal glucocorticoids on the fetal brain in the setting of chorioamnionitis are unknown. We hypothesized that antenatal glucocorticoids would modulate the inflammatory response in the fetal brain and prevent subsequent grey and white matter injury after intra-amniotic lipopolysaccharide (LPS) exposure**.**

Methods Time-mated ewes received an intra-amniotic injection of LPS and/or maternal intra-muscular betamethasone or saline, 7 and/or 14 days before delivery at 120 days gestational age (term=150d). T2-weighted MRI images were used for volumetric measurements of brain structures. The subcortical white matter (SCWM), periventricular white matter (PVWM) and hippocampus were analyzed for microglia, astrocytes, apoptosis, proliferation, myelin and pre-synaptic vesicles.

Results LPS and/or glucocorticoid exposure did not alter volume of brain structures measured on MRI. Antenatal glucocorticoids alone did not alter any of the measurements. Intra-amniotic LPS induced an inflammatory response as indicated by increased microglial and astrocyte activation which was paralleled by increased apoptotic cell death and hypomyelination in the SCWM and decreased synaptophysin density in the hippocampus. Glucocorticoid administration before the LPS exposure prevented microglial activation and the decrease in synaptophysin. Glucocorticoid treatment post LPS exposure increased microglial activation and apoptosis.

Conclusion Intra-uterine LPS exposure induced an inflammation and injury response in the fetal brain. Antenatal glucocorticoids did not alter the inflammatory changes in the brain caused by pre-existing intra-amniotic inflammation. Antenatal glucocorticoids prior to LPS reduced the effects of intra-uterine inflammation on the brain. The timing of glucocorticoid administration in the settings of intra-uterine inflammation can alter outcomes for the fetal brain.

Introduction

Preterm birth, defined as birth before 37 weeks of gestation, remains one of the largest health care problems in the Western world as up to 13% of all live births are born preterm(10, 27). Preterm birth is associated with chorioamnionitis, an infection/inflammation of the amniotic fluid and placental membranes, which is present in up to 60% of early gestation preterm births(28, 50). Exposure to intra-uterine inflammation is associated with adverse effects on fetal lung(31, 35), gut(9, 56) and brain(52) development and increases the risk for complications in postnatal life(23). Administration of antenatal glucocorticoids to induce fetal lung maturation has become standard of care to mothers at risk of preterm delivery, irrespective of the cause of preterm birth(8, 51). As chorioamnionitis is often clinically silent prior to preterm labor, combined exposures to antenatal glucocorticoids and chorioamnionitis are very common in preterm fetuses. Clinical studies show a clear relationship between the presence of chorioamnionitis and the development of neurological complications in preterm infants(19, 43). Exposure to intra-uterine inflammation can lead to intraventricular hemorrhage and white matter injury (WMI) which can manifest clinically as periventricular leukomalacia (PVL) or cerebral palsy (CP)(5, 29). Epidemiological studies also suggest a link between chorioamnionitis and an increased risk for other adverse neurodevelopmental complications such as cognitive impairments(32), autism spectrum disorders(30, 42) and schizophrenia(12, 24). However, as chorioamnionitis has a very variable presentation, neurodevelopmental outcomes after exposure to this inflammation vary greatly(11, 48).

Animal models of intra-uterine inflammation have helped to confirm clinical observations and to reveal some of the molecular mechanisms underlying fetal brain injury(3, 13, 58). Chronic intra-amniotic administration of lipopolysaccharide (LPS) to fetal sheep induced microglial activation and white matter injury, evident by loss of oligodendrocytes and hypomyelination in the subcortical white matter (SCWM)(46). Gavilanes et al. [28,29] showed that a single intra-amniotic bolus of LPS resulted in microglial activation, astrocyte proliferation and increased apoptosis in the ovine fetal brain, which were associated with functional changes in EEG after preterm birth.

The effects of intra-uterine inflammation are not limited to the brain(23). We showed that intra-amniotic LPS exposure in fetal sheep induced lung inflammation and activation of the fetal immune system(38-40). Antenatal glucocorticoid administration to the mother either before or after the LPS exposure attenuated inflammation and injury in the fetal lung and reduced the fetal immune response(18, 39). In the current study, we hypothesized that antenatal glucocorticoids would modulate the inflammatory response in the fetal brain and prevent subsequent grey and white matter injury after intra-amniotic lipopolysaccharide (LPS) exposure. For this purpose, fetal sheep were exposed *in utero* to intra-amniotic LPS and/or maternal betamethasone before preterm delivery. The effect of the timing of antenatal glucocorticoid administration in the setting of chorioamnionitis was evaluated by

administration of maternal betamethasone either before the LPS exposure (i.e. glucocorticoid pre-treatment) or after the LPS exposure (i.e. glucocorticoid post-treatment). We performed volumetric analysis of MRI images of the fetal brain and examined histological markers of brain inflammation and injury in different brain regions.

Materials and methods

Animal study

All studies were approved by the Animal Ethics Committees at The University of Western Australia and Cincinnati Children`s Hospital Medical Center. The experimental design of this animal study was published previously(40). Briefly, time-mated ewes with singleton fetuses received one of the following exposures: an intra-amniotic (IA) injection of 10 mg LPS (*Escherichia Coli*, 055:B5 Sigma-Aldrich, St. Louis, MO, USA) at 106 days gestational age (GA) (14 day LPS group) or 113 days GA (7 day LPS group), an intra-muscular injection of the corticosteroid betamethasone (Celestone Soluspan 0.5 mg/kg maternal weight, Schering-Plough, North Ryde, New South Wales, Australia) at 113 days GA (7d Beta group), a control saline injection (control group) or a combination thereof at 106 and/or 113 days GA (14 day Beta + 7 day LPS group; 14 day LPS + 7 day Beta group). Lambs were delivered by caesarean section at 120 days GA (term = 150 days GA) and euthanized at birth. The gestational age of 120 days is comparable to about 32 weeks of human gestation in terms of brain development(4). The fetal brain was perfusion-fixated via the carotid arteries with 4% paraformaldehyde for 24 hours and then stored in 30% sucrose at 4°C.

MRI analysis

Anatomical T2-weighted images were acquired using a 7 Tesla Bruker Biospin 70/30 USR scanner (Bruker, Ettlingen, Germany). After optimization all T2-weighted images were acquired using spin-echo sequences with the following parameters: (TR=2000 and TE=78 ms), with an isovolumetric voxel size of 200 μm^3 and no gap. The field of view (FOV) was 55x70x45 mm and scan matrix size 275x512x225 mm. MRI data were processed using the MIPAV software package (Medical Image Processing, Analysis, and Visualization, version 5.2.1; National Institutes of Health), which enables quantitative analysis and visualization of medical images. A blinded observer measured size and volume of several brain structures in T2-weighted images on fixation artefact free slices. Measurements included: (1) mid-sagittal antero-posterior brain diameter (mm), (2) mid-sagittal cranio-caudal diameter of the cerebellum (mm), (3) mid-sagittal antero-posterior diameter of the pons (mm), (4) mid-sagittal antero-posterior length of the corpus callosum (mm), (5) mid-sagittal volume of the corpus callosum (mm^3) and (6) maximal parasagittal volume of thalamus (mm^3) (Figure 1).

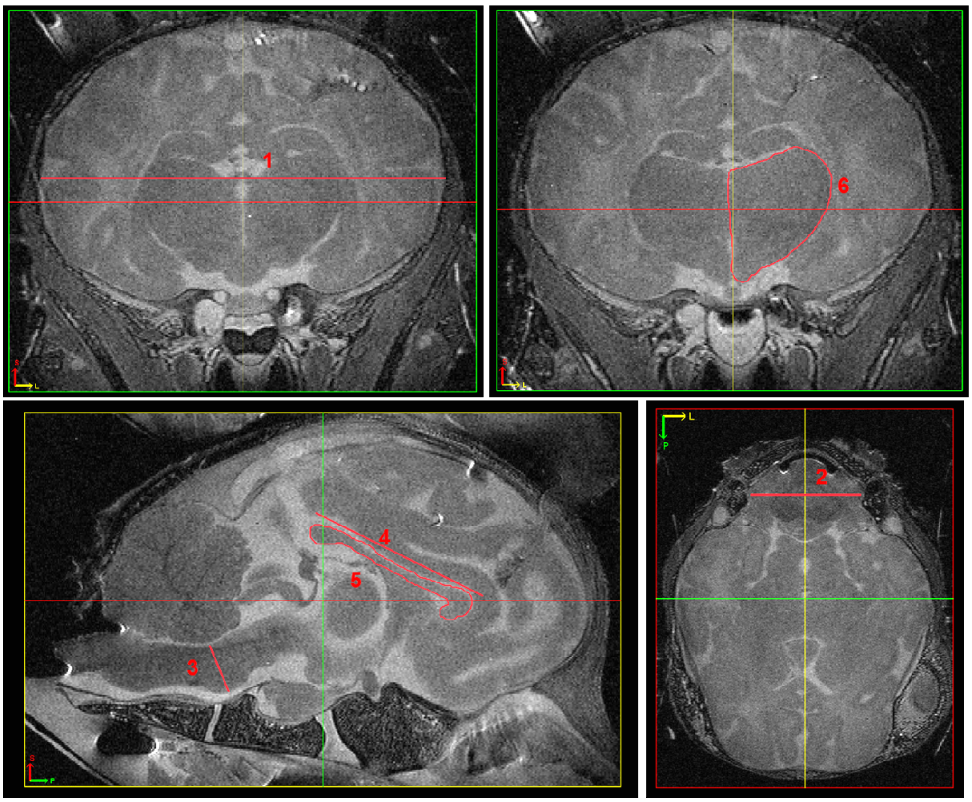

Figure 1: Volumetric measurements on T2-weighted MRI images. The mid-sagittal antero-posterior brain diameter (mm) (1), mid-sagittal cranio-caudal diameter of the cerebellum (mm) (2), mid-sagittal antero-posterior diameter of the pons (mm) (3), mid-sagittal antero-posterior length of the corpus callosum (mm) (4), mid-sagittal volume of the corpus callosum (mm^3) (5) and maximal parasagittal volume of thalamus (mm^3) (6) of the ovine fetal brain was measured on T2-weighted MRI images.

Immunohistochemistry

For immunohistochemistry of the fetal brain, tissue from the right hemisphere was embedded in 10% gelatin. Serial coronal sections (50 µm) containing the posterior hippocampus were cut on a vibrotome (Leica Biosystems, Nussloch, Germany). Sections were stained by free-floating techniques as described previously(33) for ionized calcium binding adaptor molecule 1 (IBA1, #019-19741, Wako Pure Chemical Industries, Osaka, Japan), glial fibrillary acidic protein (GFAP, Z0334, Dakocytomation, Glostrup, Denmark) cleaved caspase-3 (Asp175, #9661S, Cell Signaling Technology, Boston, USA), Ki67 (M7240, Dakocytomation), myelin basic protein (MBP, MAB386, Merck Millipore, Billerica, MA, USA) and synaptophysin (MAB5258, Merck Millipore). Briefly, sections were rinsed with Tris buffered saline (TBS, pH 7.6) and TBS-Triton (TBS-T, pH 7.6). Endogenous peroxidase activity was blocked by incubating in 0.3% H_2O_2 in TBS. Next, the sections were incubated overnight at 4°C with the diluted primary antibody (IBA1 1:1000, GFAP 1:2000, cleaved caspase-3 1:800, Ki67 1:100, MBP 1:2000, synaptophysin 1:2000 with 0.3% donkey serum). After rinsing, a secondary donkey-anti-rabbit (IBA1, GFAP, cleaved caspase-3), donkey-anti-mouse (synaptophysin, Ki67) or donkey-anti-rat (MBP) biotin labeled antibody was added for

incubation in room temperature for 2 hours. The immunostaining was enhanced with Vectastain ABC peroxidase Elite kit (PK-6200, Vector Laboratories, Burlingame, USA) followed by nickel sulfate-diaminobenzidine. The stained sections were mounted on gelatin-coated glass slides, dehydrated and coverslipped.

Quantification

For the analysis of IBA1 and GFAP immuno-reactivity in the hippocampus, one digital image per coronal section (n=5 sections per animal) of the dentate gyrus (DG), cornu ammonis (CA)1/2, CA3 and CA4 were acquired at 100x magnification with an Olympus AX-70 microscope connected to a digital camera (F-view, Olympus, Tokyo, Japan). In the SCWM and PVWM two images per coronal section (n=5 sections per animal) were obtained on a standardized location within these regions of interest. All images were collected under the same lighting conditions. The area fraction (%) of IBA1 or GFAP immuno-reactivity was measured by applying a standard threshold using a specifically designed algorithm in Leica QWin Pro V 3.5.1 software (Leica, Rijswijk, the Netherlands)(33).

To measure the density (cells per mm^2) of Ki67-positive cells and cleaved caspase-3 positive cells, digital images of the hippocampus (4 images), PVWM (2 images) and SCWM (2 images) in the standardized regions described above on 5 consecutive coronal sections per animal were taken at 200x magnification with the same microscope and digital camera as above. Ki67-positive cells were counted using Image J software (Rasband, W.S., Image J US National Institutes of Health, Bethesda, Maryland, USA). The density (cells per mm^2) of cleaved caspase-3 positive cells in the PVWM and SCWM were similarly measured. For the analysis in the hippocampus, the area fraction (%) of cleaved caspase-3 immuno-reactivity was measured using Leica QWin Pro V 3.5.1 software, since the hippocampus is densely populated with overlapping cleaved caspase-3 positive cells that could not be distinguished as single cells(57). For the analysis of MBP and synaptophysin, digital images from the SCWM (2 images) for MBP and from the hippocampus (2 images in CA1/2 region, 2 images in CA3, 1 image in CA4 and DG) for the synaptophysin staining on 5 consecutive coronal sections per animal were acquired similarly at 200x magnification. In the PVWM no MBP immuno-reactivity was detected indicating that this white matter region was not myelinated(33). The area fraction (%) of MBP and synaptophysin immuno-reactivity was measured using Leica QWin Pro V 3.5.1 software. All described analyses were performed by a blinded observer.

Data analysis

Data are expressed as mean ± SEM. For the cleaved caspase-3 analysis, data are transformed to fold change compared to control in order to compare the results in the different regions. Groups are compared using one-way ANOVA with Tukey or Dunnett's for post-hoc analysis or by a non-parametric Kruskal-Wallis test as appropriate. Statistical analysis was performed by GraphPad Prism v5.0. Significance was accepted at $p<0.05$.

Results

MRI measurements

T2-weighted MRI images were used to measure anatomical variables such as brain diameter, cerebellum and pons diameter, corpus callosum (CC) length and CC and thalamus volume (Figure 1). Exposure to intra-amniotic LPS and/or maternal antenatal glucocorticoids did not alter any of the measurements in the fetal brain compared to control animals (Table 1).

Table 1: Volumetric data measured on MRI images

	Control (n=4)	7d Beta (n=6)	7d LPS (n=7)	14d LPS (n=7)	14d Beta + 7d LPS (n=6)	14d LPS + 7d Beta (n=7)
Brain (mm)	20.0±1.1	18.4±0.9	23.4±3.7	19.2±0.7	18.4±0.7	22.5±0.5
Cerebellum (mm)	8.7±0.5	8.2±0.4	10.2±1.9	8.5±0.2	9.0±0.6	10.1±0.2
Pons (mm)	2.3±0.2	2.2±0.1	2.7±0.4	2.3±0.1	2.3±0.1	2.8±0.1
Corpus callosum (mm)	8.1±0.3	7.9±0.2	11.2±2.3	7.9±0.4	8.0±0.6	9.2±0.4
Corpus callosum (mm^3)	2.8±0.4	2.7±0.2	4.2±1.3	3.1±0.2	2.7±0.2	3.1±0.1
Thalamus (mm^3)	8.5±1.4	8.9±0.8	12.1±2.6	9.4±0.6	10.1±0.7	9.3±0.5

Data corrected for body weight and expressed as mean ± SEM. LPS: lipopolysaccharide; Beta: betamethasone.

Regional alterations in the fetal brain after intra-amniotic LPS exposure and antenatal glucocorticoids

Brain inflammation was investigated by measuring the area fraction (%) of IBA1 and GFAP as markers for microglial and astrocyte activation respectively, in the fetal hippocampus (Figure 2), subcortical white matter (Figure 3) and periventricular white matter (Figure 4). Apoptosis was assessed by immunohistochemical staining for cleaved caspase-3, proliferating cells were identified by Ki67 staining, myelination shown by MBP and pre-synaptic vesicle density was assessed by synaptophysin staining.

Hippocampal changes

Administration of antenatal glucocorticoids alone did not alter IBA1 (Figure 2A) or GFAP (Figure 2F) immuno-reactivity in the fetal hippocampus nor did it induce increased apoptosis or change proliferation rates (Table 2). No changes in synaptophysin immuno-reactivity were detected after glucocorticoid treatment only (Figure 2K).

Exposure to intra-amniotic LPS 7 days before delivery increased hippocampal IBA1 immuno-reactivity (Figure 2A). Microglia of LPS exposed animals (Figure 2C) showed several morphological changes such as hypertrophic cell bodies and thickened shortened processes compared to controls (Figure 2B). GFAP immuno-reactivity increased significantly in the hippocampus of animals exposed to LPS 7 or 14 days before delivery (Figure 2F). Compared to controls (Figure 2G), GFAP-positive cells in the LPS exposed animals (Figure 2H) showed enlarged cell bodies and retracted processes suggesting active gliosis. Exposure to LPS 7 or 14 days before delivery decreased the number of Ki67-positive cells/mm^2 and increased apoptosis by two-fold in the fetal hippocampus compared to controls (Table 2). Both 7 day and 14 day intra-amniotic LPS exposure significantly decreased the area fraction (%) of synaptophysin immuno-reactivity (Figure 2K). LPS exposed animals (Figure 2M) showed a clear reduction in synaptophysin immuno-reactivity in the fetal hippocampus compared to controls (Figure 2L).

Glucocorticoid treatment followed by intra-amniotic injection of LPS (14d Beta + 7d LPS) reduced IBA1 immuno-reactivity in the fetal hippocampus compared to 7d LPS exposed animals (Figure 2D). GFAP immuno-reactivity in the 14d Beta + 7d LPS animals (Figure 2I) remained increased. Glucocorticoid treatment before the LPS exposure still resulted in increased apoptosis but normalized the proliferation rate (Table 2) and synaptophysin immuno-reactivity (Figure 2K, 2N) in the hippocampus. Animals exposed to intra-amniotic LPS followed by betamethasone treatment (14d LPS + 7d Beta) showed increased IBA1 immuno-reactivity in the fetal hippocampus compared to animals only exposed to LPS 14 days before delivery (Figure 2E). Hippocampal proliferation, apoptosis (Table 2) and synaptophysin immuno-reactivity (Figure 2O) normalized in 14d LPS + 7d Beta animals.

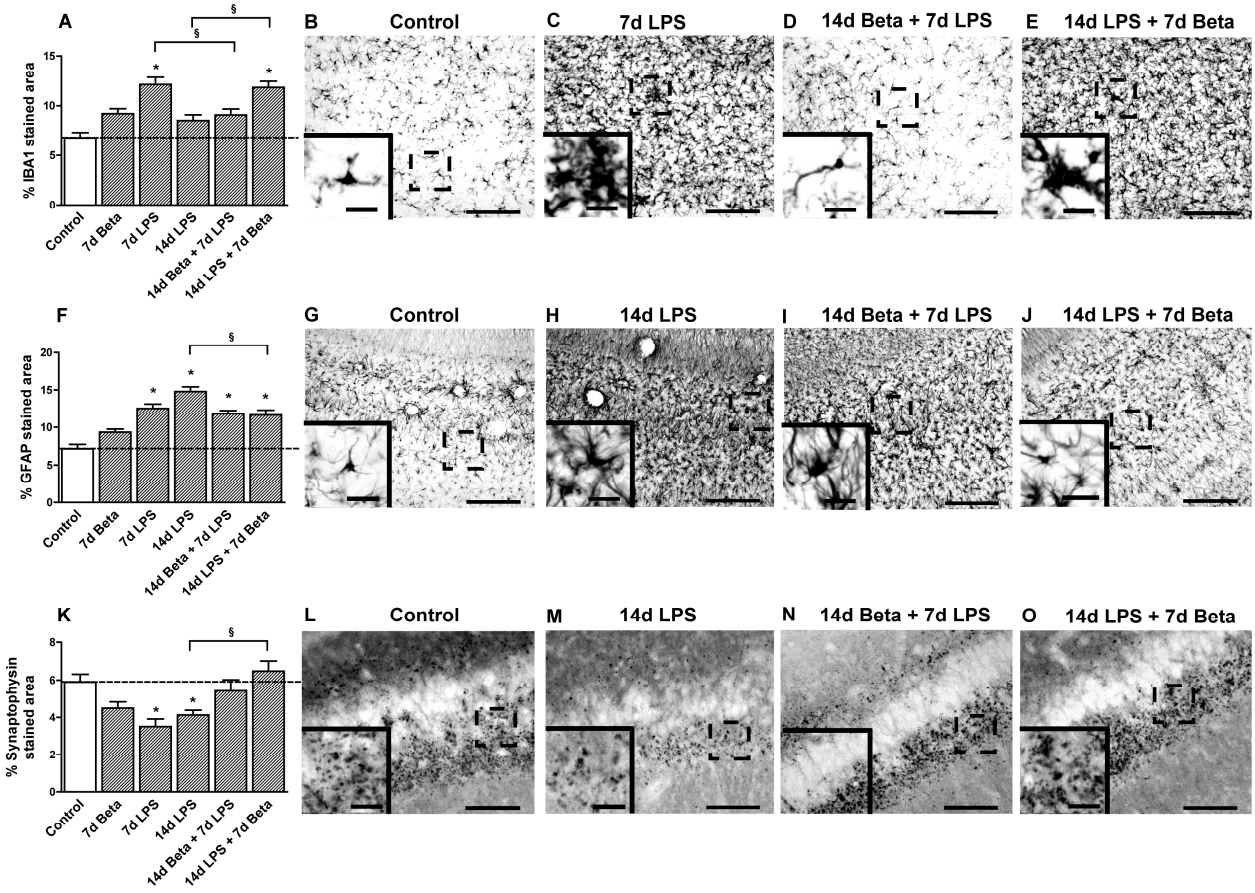

Figure 2: Hippocampal changes after intra-amniotic LPS exposure and antenatal glucocorticoids. A: The area fraction (%) of IBA1 immuno-reactivity increased significantly after intra-amniotic LPS exposure 7 days before delivery and combined 14 day LPS and 7 day glucocorticoid exposure. B-E: Representative images of the IBA1 staining in the CA1 region of the hippocampus in controls (B), 7d LPS (C), 14d Beta + 7d LPS (D) and 14d LPS + 7d Beta (E) exposed animals. F: Intra-amniotic LPS exposure significantly increased the area fraction (%) of GFAP immuno-reactivity in all experimental groups. G-J: Representative images of the GFAP staining in the CA1 region of the hippocampus in controls (G), 14d LPS (H), 14d Beta + 7d LPS (I) and 14d LPS + 7d Beta (J) exposed animals. K: The area fraction (%) of synaptophysin immuno-reactivity decreased both 7 and 14 days after intra-amniotic LPS exposure compared to controls. L-O: Representative images of the synaptophysin staining in the CA1 region of the hippocampus in controls (L), 14d LPS (M), 14d Beta + 7d LPS (N) and 14d LPS + 7d Beta (O) exposed animals. Scale bar = 200 µm; scale bar insert = 25 µm. *$p<0.05$ versus controls and § $p<0.05$ between experimental groups using a one-way ANOVA with Tukey's post hoc test.

Table 2: Antenatal corticosteroid treatment modulates the LPS-induced effects on apoptosis and proliferation in the fetal brain

	Control	7d Beta	7d LPS	14d LPS	14d Beta + 7d LPS	14d LPS + 7d Beta
Ki67 (cells/mm^2)						
Hippocampus	126±8	136±11	91±8*	58±6*	135±21	145±7$^{£}$
PVWM	71±9	71±11	54±5	66±10	55±15	70±6
SCWM	80±7	95±11	49±6	58±7	71±10	87±5
Cleaved caspase-3 (fold change)						
Hippocampus	1±0.1	1.2±0.1	2.1±0.1*	1.9±0.1*	1.7±0.1*	1.5±0.1
PVWM	1±0.1	0.9±0.1	1.4±0.1*	0.8±0.1	0.9±0.3§	1.5±0.3*$^{£}$
SCWM	1±0.1	1.6±0.1	1.4±0.2	1.4±0.2	1.3±0.1	2.1±0.2*

Data expressed as mean ± SEM. LPS: lipopolysaccharide; Beta: betamethasone. * $p<0.05$ versus controls; § $p<0.05$ versus 7d LPS; £ $p<0.05$ versus 14d LPS

White matter changes

Antenatal glucocorticoid treatment 7 days before delivery did not affect any of the measurements in the fetal subcortical (Figure 3) and periventricular white matter (Figure 4). Exposure to LPS increased IBA1 and GFAP immuno-reactivity both in the SCWM (Figure 3A, F) and PVWM (Figure 4), with morphological changes consistent with activation of microglia and astrocytes (Figure 3C). Proliferation rates were unchanged in the white matter and apoptosis was slightly increased in the PVWM in animals exposed to LPS 7 days before delivery (Table 2). MBP immuno-reactivity in the SCWM was investigated as an indication of white matter injury. Control animals had abundant MBP-positive sheaths and MBP-positive cells in the SCWM (Figure 3L). Animals exposed to LPS showed less MBP immuno-reactivity and loss of MBP-positive cells (Figure 3K, M) indicating white matter injury in the SCWM.

Animals exposed to 14d Beta + 7d LPS showed reduced IBA1 immuno-reactivity in the SCWM (Figure 3A, D) and reduced apoptosis in the PVWM (Table 2) compared to 7 day LPS exposed animals. GFAP immuno-reactivity in the 14d Beta + 7d LPS animals remained as high in the white matter as that seen in the 7 day LPS exposed animals (Figure 3F, 4B).

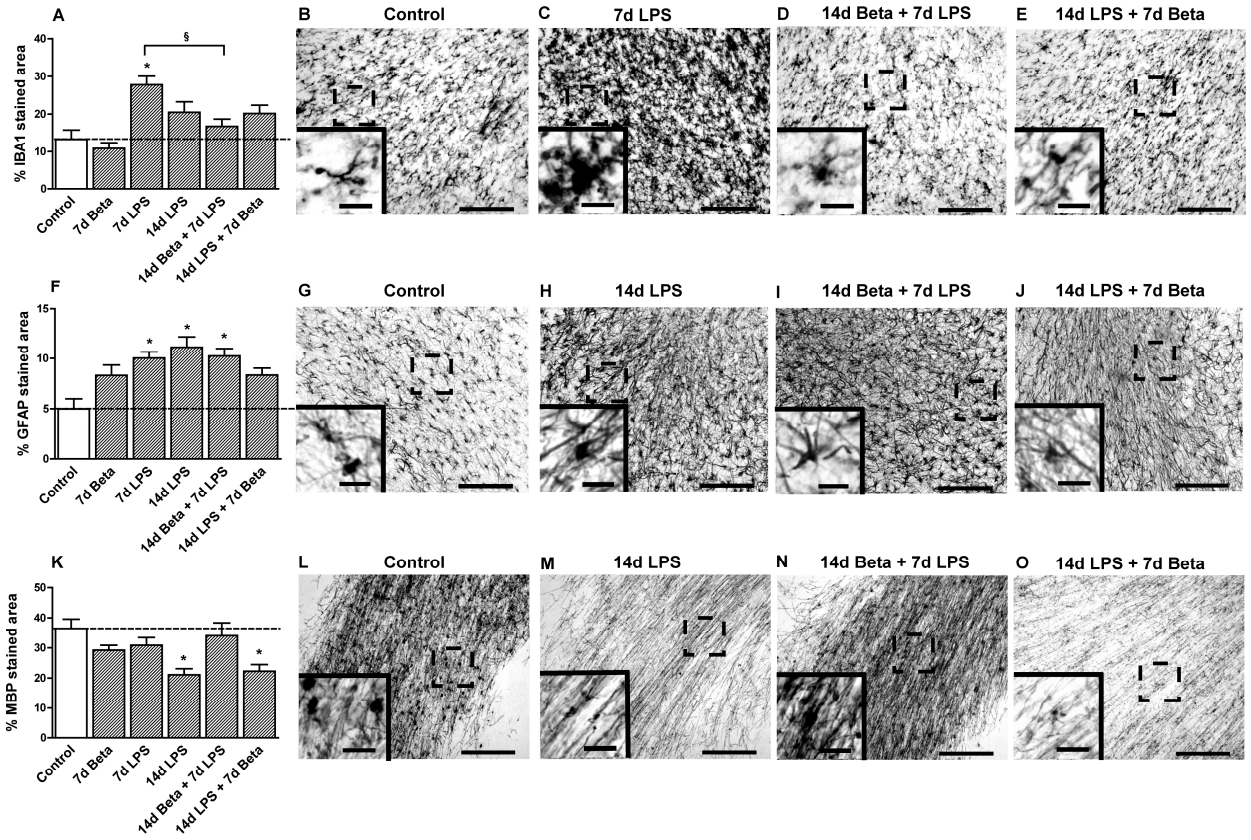

Figure 3: Effect of intra-amniotic LPS and antenatal glucocorticoids on the subcortical white matter. A: The area fraction (%) of IBA1 immuno-reactivity increased significantly after intra-amniotic LPS exposure 7 days before delivery. B-E: Representative images of the IBA1 staining in the SCWM in controls (B), 7d LPS (C), 14d Beta + 7d LPS (D) and 14d LPS + 7d Beta (E) exposed animals. F: LPS exposure 7 or 14 days before delivery significantly increased the area fraction (%) of GFAP immuno-reactivity. G-J: Representative images of the GFAP staining in the SCWM in controls (G), 14d LPS (H), 14d Beta + 7d LPS (I) and 14d LPS + 7d Beta (J) exposed animals. K: The area fraction (%) of MBP immuno-reactivity decreased 14 days after intra-amniotic LPS exposure irrespective of glucocorticoid treatment compared to controls. L-O: Representative images of the MBP staining in the SCWM in controls (L), 14d LPS (M), 14d Beta + 7d LPS (N) and 14d LPS + 7d Beta (O) exposed animals. Scale bar = 200 µm; scale bar insert = 25 µm. *$p<0.05$ versus controls and § $p<0.05$ between experimental groups using a one-way ANOVA with Tukey's post hoc test.

Compared to animals which were only exposed to LPS 14 days before delivery, animals exposed to 14d LPS + 7d Beta showed increased IBA1 immuno-reactivity in the PVWM (Figure 4A) and induced apoptosis in the SCWM and PVWM (Table 2). Glucocorticoid treatment after the LPS exposure decreased GFAP immuno-reactivity (Figure 3F, 4B). White matter injury was still evident in the 14d LPS + 7d Beta animals (Figure 3O) with decreased MBP immuno-reactivity in the SCWM.

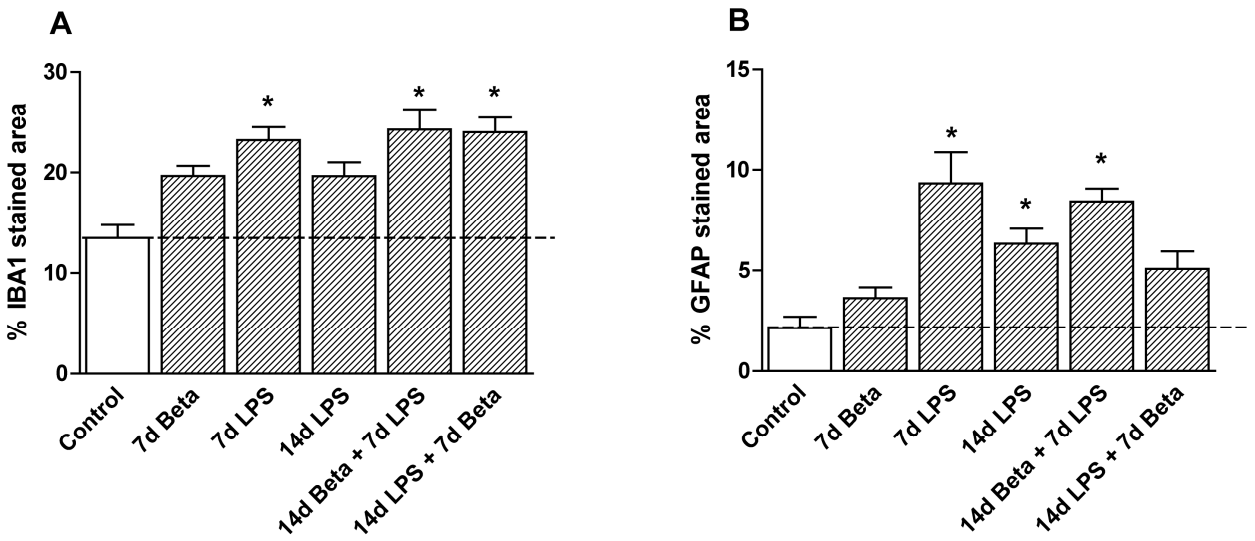

Figure 4: Periventricular white matter changes after intra-amniotic LPS and antenatal glucocorticoids. A: The area fraction (%) of IBA1 immuno-reactivity in the PVWM increased significantly in animals exposed to intra-amniotic LPS 7 days before delivery and in animals that were exposed to both LPS and glucocorticoids. B: LPS exposure 7 or 14 days before delivery significantly increased the area fraction (%) of GFAP immuno-reactivity. Animals exposed to 14d Beta + 7d LPS also showed increased GFAP immuno-reactivity in the PVWM. *p<0.05 versus controls and § p<0.05 between experimental groups using a one-way ANOVA with Tukey's post hoc test.

Discussion

We investigated the effects of intra-uterine inflammation and antenatal glucocorticoids, two common antenatal exposures for preterm infants, on the ovine fetal brain. Intra-amniotic LPS exposure induced a cerebral inflammatory response with subsequent grey and white matter injury. Glucocorticoid treatment in the absence of intra-uterine inflammation did not affect the fetal brain. Antenatal maternal glucocorticoids administered with pre-existing intra-amniotic inflammation did not prevent the inflammatory changes in the fetal brain. Antenatal glucocorticoids given prior to LPS reduced the effects of intra-uterine inflammation on the fetal brain. This study demonstrates that the timing of glucocorticoid administration can alter the LPS-mediated effects on the fetal brain.

Our findings did not show any harmful effects of antenatal glucocorticoid treatment in the absence of intra-uterine inflammation on the fetal brain. Although antenatal glucocorticoids reduce the risk of intraventricular hemorrhage and periventricular leukomalacia(6, 8), there are concerns about the effects of glucocorticoids on fetal brain development, in particular on brain growth(22, 45), neurogenesis(47, 54) and myelination(21). Recently, Tijsseling et al.(53) showed reduced histological neuronal density in the hippocampus of neonates after antenatal glucocorticoid treatment. Data from experimental animal models show that multiple doses of antenatal glucocorticoids at different time points during gestation can modulate apoptotic rates(44). Furthermore, repeated doses of antenatal glucocorticoids

decrease the expression of cytoskeletal proteins microtubule-associated protein (MAP)1B and MAP2 and synaptophysin expression in fetal sheep brain suggesting functional disturbances in the neuronal network(1, 2). Although it is less clear how a single course of antenatal glucocorticoids affects the developing brain, the beneficial effects of glucocorticoids on lung maturation and consequent neonatal survival outweigh these concerns.

We used T2-weighted MRI imaging to investigate volumetric changes in the ovine fetal brain. The MRI imaging did not detect any gross anatomical changes after exposure to intra-amniotic LPS which is in line with previous clinical findings(16, 36). Exposure to intra-uterine inflammation induced an inflammatory response in the fetal brain as indicated by increased microglial activation and astrogliosis associated with hypomyelination and reduced synaptophysin expression. We did not monitor brain function, but microglial activation and apoptosis in the white matter of preterm lambs exposed to LPS correlated with increased EEG delta frequency which was associated with seizure disorders(25, 26). Studies of Dean et al.(20) and Keogh et al.(37) support these findings by showing that inflammatory changes in the ovine fetal brain correlated with loss of the normal maturational increase in the EEG amplitude. Furthermore, our findings of decreased pre-synaptic vesicle density in the fetal hippocampus suggest that synaptic transmission was compromised after LPS exposure(55).

We investigated the effect of the timing of combined exposure to intra-amniotic inflammation and antenatal glucocorticoids on the ovine preterm brain, since glucocorticoids can have both pro- and anti-inflammatory properties in the presence of inflammation(15). Antenatal glucocorticoids are administered when preterm birth is imminent irrespective of the cause of preterm birth(8). However, there is a lack of knowledge concerning the use of antenatal glucocorticoids in the settings of intra-uterine infections(8). Kallapur et al.(34) previously showed that combined maternal betamethasone and intra-amniotic LPS exposure increased fetal lung inflammation compared to LPS exposure alone suggesting that glucocorticoids alter the immune response of the preterm lung to inflammation. In this model antenatal exposure to glucocorticoids either before or after an inflammatory challenge has contrasting effects on the fetal immune response(39, 40) and subsequent brain inflammation and injury. Although the anti-inflammatory actions of glucocorticoids have been recognized for several decades, recent studies suggest that glucocorticoids can also have pro-inflammatory properties(15, 49). The balance of these pro- and anti-inflammatory effects depends on which phase of the immune response the glucocorticoids are administered. Glucocorticoid-mediated activation of the glucocorticoid receptor induces suppression of transcriptional activity of NF-κB and other genes involved in the inflammatory response(7, 41). Therefore pre-treatment with glucocorticoids inhibits the subsequent inflammatory response initiated by LPS-mediated Toll-like receptor signaling, resulting in anti-inflammatory immunological response. However, when the inflammatory response is initiated before activation of the glucocorticoid receptor, the immune response may shift to pro-inflammatory as glucocorticoids can enhance the expression of Toll-like

receptors and consequently increase pro-inflammatory cytokine production(14, 17). Consequently, the timing of exposure to anti- and pro-inflammatory stimuli appears to be essential in determining the eventual outcome of the immune response, not only *in vitro*, but also *in vivo*.

There are limitations to this study. We were not able to identify which cells in the fetal brain underwent apoptosis or loss of proliferation after the exposures. Furthermore, we did not assess what the effects of these histological changes were on the functional outcome of the fetal brain. Animals in this study were exposed to intra-amniotic LPS and maternal glucocorticoids either 7 days or 14 days before preterm delivery. However, exposures at different time points, for different intervals and to a single dose or repeated doses of glucocorticoids during fetal development may have different outcomes. Although our study does not identify new concerns for the use of a single dose of glucocorticoids, long term effects of antenatal glucocorticoid treatment on neonatal neurodevelopment remain unclear.

In conclusion, we have shown that intra-amniotic LPS exposure induced inflammation and injury in ovine preterm brain without changes on MRI. Our findings demonstrate that maternal antenatal betamethasone administration does not prevent nor potentiate the inflammatory changes in the fetal brain caused by pre-existing intra-amniotic inflammation. Antenatal glucocorticoids given prior to intra-amniotic inflammation reduced the cerebral inflammatory response after intra-amniotic LPS and prevented grey and white matter injury in the fetal brain. This study shows that the timing of treatment with maternal glucocorticoids in the setting of intra-uterine inflammation determines the effects on the preterm brain. Therefore, future experimental studies should focus on the immuno-modulatory effects of antenatal glucocorticoid treatment at the onset of chorioamnionitis.

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Chapter 9

Summary and General Discussion

Chorioamnionitis and maternal glucocorticoids are two highly common antenatal exposures in preterm babies which are linked to beneficial and adverse outcomes for the preterm infant depending on the type of exposure, timing of exposure during gestation, the organ in question, the gestational age at birth etc. (9, 13). In addition, the effect of the combined exposure to maternal glucocorticoids and chorioamnionitis, which is very common in preterm infants, is not well understood. Therefore, the aim of this present thesis was to explore how the combined exposure to maternal glucocorticoids and intra-amniotic inflammation affected the fetal lungs, brain and immune system in a well-defined animal model that is appropriate with respect to organ development (66).

The multi-organ effects of chorioamnionitis

It has been established in the last years that chorioamnionitis induces a multi-organ disease of the fetus, affecting nearly all organ systems ranging from the lungs, gut and brain to the skin, immune and cardiovascular system (27). In this thesis, we further characterized the effects of intra-amniotic exposure to LPS on the lungs, brain, thymus and spleen of preterm lambs in order to unravel some of the molecular mechanisms which associate chorioamnionitis and/or antenatal glucocorticoids with adverse neonatal outcomes in preterm infants (Figure 1).

In **chapter 2, 3** and **4** the effects of intra-amniotic LPS exposure on pulmonary inflammation, maturation and lung development were assessed. A single intra-amniotic injection of LPS induced pulmonary inflammation up to 14 days after administration. This pulmonary inflammatory response was accompanied by lung maturation as illustrated by increased production of surfactant proteins and improved lung compliance indicating the link between exposure to chorioamnionitis and a decreased incidence of RDS in preterm infants (12). However, the presence of chorioamnionitis is also associated with an increase in BPD in preterm babies, due to an arrest in late lung development.

Therefore we examined the effects of intra-amniotic LPS exposure on important lung developmental pathways Shh and Wnt signaling in **chapter 3** and **4**. We showed that exposure to inflammation resulted in decreased Shh and Wnt signaling which can form the basis for aberrant lung development. Future in vitro studies are needed to demonstrate this causal link between the presence of inflammation and disturbances in these pathways and subsequently late lung development.

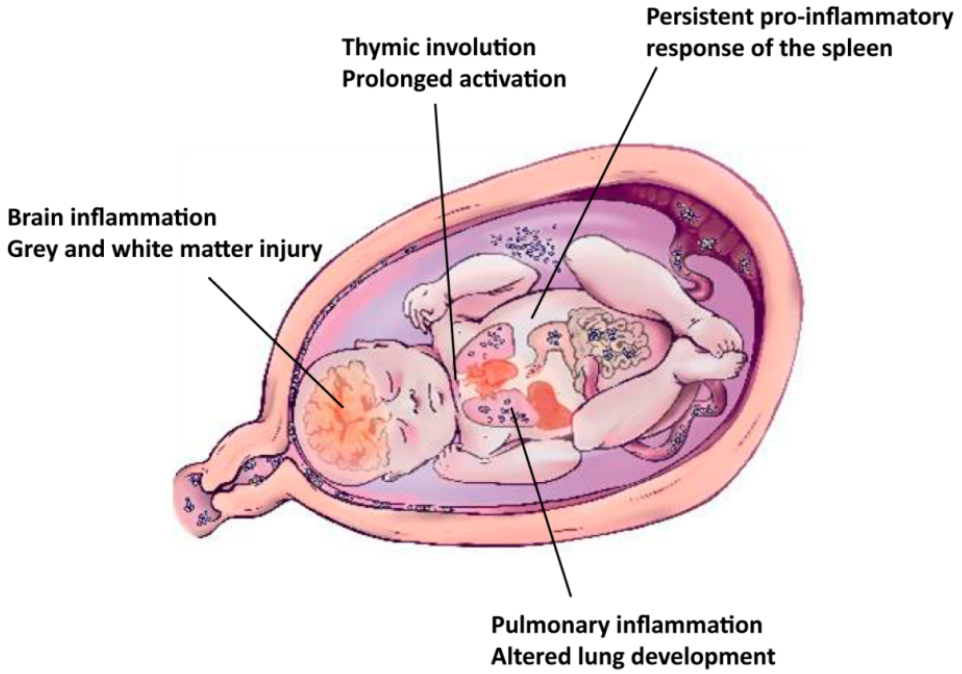

Figure 1: Summary of the changes in fetal organs after exposure to intra-amniotic LPS as described in this thesis (adjusted from (27)).

However, this new information uncovers interesting options for possible intervention strategies as several modulators of these pathways are available (20, 34, 56). Shh signaling can be activated or inhibited by various pharmacological modulators such as purmorphamine (Shh agonist) and cyclophamine (Shh antagonist) respectively, in order to re-direct of re-stimulate late lung development (42, 62). Recent studies showed that acute lung injury due to LPS exposure in adult mice was worsened by inhibition of Shh signaling by cyclophamine administration but reduced by addition of recombinant Shh (67). Also re-directing Wnt signaling in the fetal lungs may provide beneficial outcomes for the premature lung. Inhibition of GSK3-β by a pharmacological modulator SB216763, resulting in activation of canonical Wnt signaling, reduced pulmonary inflammation and improved alveolarization in a hyperoxia rodent model of BPD (32). These beneficial effects of Wnt activation on lung repair and development after injury are possibly in part modulated by differentiation of endogenous mesenchymal stem cells to alveolar type II cells which has been shown in vitro (44). Future studies will be needed to show if and how pharmacological modulation of the Shh and Wnt pathway can re-direct late lung development after exposure to chorioamnionitis.

Antenatal exposure to inflammation may also affect neonatal outcome by modulating the function of the fetal immune system. Both hypo- and hyper-responsiveness of the immune response can potentially induce organ damage and may lead to the increased susceptibility for postnatal infections and sepsis in preterm infants (3) (Figure 3). Although clinical studies reported that exposure to chorioamnionitis can affect the fetal immunological organs (19, 24, 59), little is known about how the immunological organs respond to intra-uterine

inflammation. In **chapter 5, 6** and **7** we showed that intra-amniotic LPS exposure resulted in alterations in both the fetal thymus and spleen. The thymus (**chapter 5** and **6**) and spleen (**chapter 7**) were able to mount a pro-inflammatory response after intra-amniotic LPS exposure. Marked changes in both the cytokine profile and T-cell composition were noticeable up to 15 days after the LPS injection. This prolonged activation of the fetal immunological organs demonstrates that a single bacterial component can cause a persistent inflammatory response in the fetus (48). Intra-uterine inflammation also caused changes in the thymic micro-environment (**chapter 6**) which can result in a deregulated T-cell development and subsequent T-cell function in postnatal life (29, 45).

In addition to the pulmonary and immunological response, we also evaluated the neurological response of preterm sheep after exposure to intra-amniotic LPS in **chapter 8**. The ovine fetal brain showed signs of inflammation and injury with increased microglial infiltration and hypo-myelination, which are characteristics of white matter injury in preterm infants (4, 18). Although the relationship between intra-uterine inflammation and lung injury (30, 33) on one hand and intra-uterine inflammation and brain damage (21, 53) on the other hand has been described extensively both in clinical and experimental studies, it remains unclear how both adverse outcomes relate to each other, especially in the context of chorioamnionitis (2, 22). It is probable that an inadequate or excessive fetal immune response may underlie both the pathology in the preterm brain and lungs. In fact, deregulation of T-cell development has been associated with adverse outcomes such as BPD (23, 52), necrotizing enterocolitis (64) and cerebral white matter disease (37) in preterms suggesting that the changes in the immunological organs may play a key role in the initiation and progression of excessive inflammation and injury in the other fetal organs.

Antenatal glucocorticoids: how do the benefits outweigh the potential risks?

In 1972, Liggins and Howie reported their findings of the first randomized controlled trial of antenatal glucocorticoid treatment in case of premature delivery for the prevention of respiratory distress in preterm infants (43). These observations were soon reproduced in several human and experimental studies followed by the general practice of administration of antenatal dexamethasone or betamethasone to women at risk of preterm delivery before 34 weeks of gestation (51). Although this led to a significant increase in the neonatal survival rate, some concerns remain about the possible adverse effects of a single or multiple courses of maternal glucocorticoids on fetal growth and development (14, 26, 63). There are several controversial reports about the long term effects of antenatal exposure to glucocorticoids, which are mainly linked to adverse neurodevelopmental or immunological outcomes (26, 55). Therefore, we studied the effects of antenatal glucocorticoid exposure on the fetal lung (**chapter 2, 3** and **4**), thymus (**chapter 6**) and brain (**chapter 8**) in preterm lambs. Maternal betamethasone given 7 days before preterm delivery induced mild lung maturation (**chapter 2**) but did not affect lung developmental pathways or pulmonary

structure (**chapter 3** and **4**) neither did it result in any beneficial or harmful effects on the fetal thymus or brain suggesting that a single course of antenatal glucocorticoids does not harm fetal development in this model.

Clinical guidelines have described chorioamnionitis as a contraindication for the administration of antenatal glucocorticoids in fear of an increased risk of neonatal sepsis (10). As there is a clear lack of knowledge concerning the interactive effects of maternal glucocorticoid treatment in the setting of chorioamnionitis, we aimed to elucidate how the combination of these antenatal exposures modulated neonatal outcome. Maternal glucocorticoid administration 7 days after the exposure to intra-amniotic LPS was not able to inhibit or prevent an inflammatory response in the fetus. Signs of inflammation were noticeable in the fetal lungs (**chapter 2**), thymus (**chapter 6**) and brain (**chapter 8**) resulting in structural alterations in the lungs (**chapter 3**) and hypo-myelination in the brain. Surprisingly, when glucocorticoids were given 7 days before the inflammatory stimulus, hardly any inflammation was detected in any of the investigated organs. Antenatal betamethasone before intra-amniotic LPS exposure suppressed the fetal inflammatory response resulting in little to no changes in the fetal lungs or brain. As we also did not detect any thymic response in these animals, we speculate that maternal glucocorticoids may precondition the fetal immune system resulting in immune hypo-responsiveness or paralysis. Although this situation decreases the immediate tissue damage in the fetus and probably decreases the risk for adverse neonatal outcomes such as cerebral white matter disease and BPD, it may predispose a preterm infant to postnatal infections.

The underlying mechanisms of this preconditioning still remain unclear and require further investigation. As synthetic glucocorticoids are potent anti-inflammatory drugs, they are capable to modulate the (fetal) immune system in several ways (16, 54). They can interrupt pro-inflammatory signaling cascades, induce apoptosis of immune cells or increase transcription of anti-inflammatory factors thereby diminishing the immune response (16). In this aspect, the interaction between glucocorticoid and NF-κB signaling has been extensively characterized. Upon binding of glucocorticoids to their receptor, the glucocorticoid receptor (GR) is able to modulate the pro-inflammatory NF-κB pathway by several means. The GR can physically interaction with the p65 subunit of NF-κB thereby preventing the DNA binding of NF-κB to promotor site of target genes. The GR can indirectly repress transcription of NF-κB target genes by histon modifications or chromatin remodeling or induce upregulation of NF-κB inhibitors such as IκB (8). This extensive interaction between the GR and NF-κB signaling can result in an altered net outcome of the immune response depending on the order and the magnitude of activation of both cascades (50). Maternal glucocorticoid administration may also influence the fetal immune system indirectly by modulating the fetal hypothalamic-pituitary-adrenocortical (HPA)-axis (57). Both human and animal studies have described changes in the response of the fetal HPA axis after prenatal exposure to glucocorticoids which may be linked to a number of adverse health outcomes and diseases in later life (57, 61). Glucocorticoid exposure *in utero* results in suppression of the HPA response under basal

conditions but also after pain-related stress. These changes persisted well beyond the exposure to these glucocorticoids and may result in an altered fetal development, in particular in case of the fetal immune system (5, 6).

Altogether, these results demonstrated that the timing of glucocorticoid administration in the settings of intra-uterine inflammation altered the outcome for the investigated organs. Glucocorticoid administration before the inflammatory stimulus prevented most adverse alterations whereas glucocorticoids after the induction of inflammation only appeared to have some beneficial effects on the fetal lung. However, in both scenario`s glucocorticoid administration did not aggravate fetal tissue damage in any of the investigated organs. These findings support the recent clinical observations that maternal glucocorticoids in the settings of chorioamnionitis decrease the risk of adverse neonatal complications (10).

Implications

The eventual neonatal outcome after preterm birth is dependent on the complex interaction and the timing of exposure to antenatal events and postnatal factors (Figure 2).

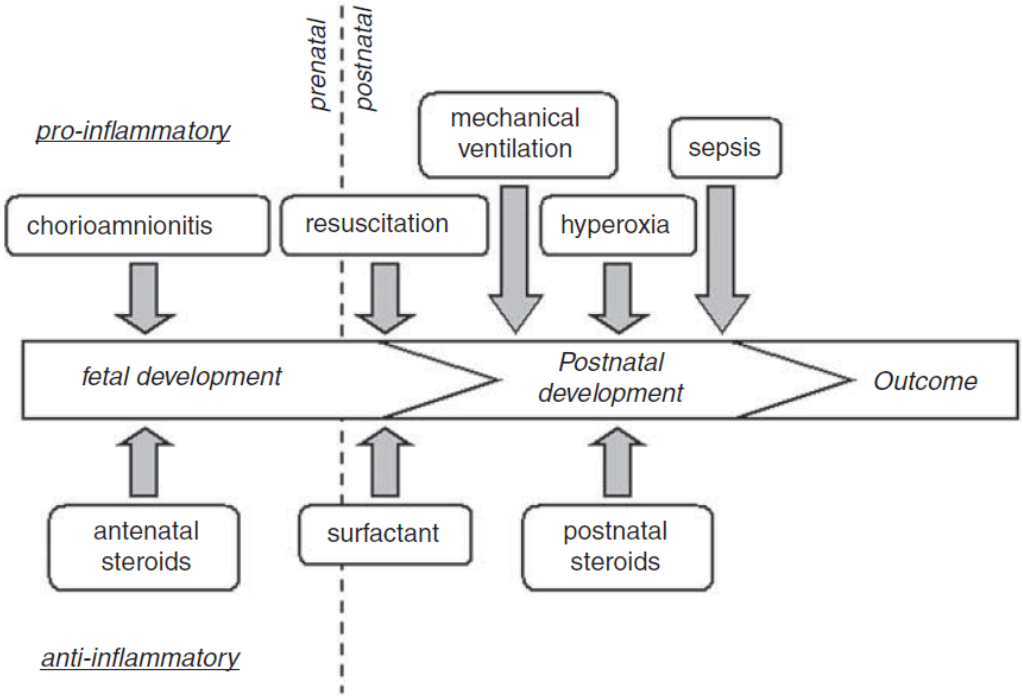

Figure 2: Overview of the most common antenatal and postnatal events to which preterm infants are exposed. Both antenatal and postnatal exposures can influence the neonatal immune response and subsequent neonatal outcome (27).

In recent years, the fetal immune response towards these perinatal events has been identified as a key determinant for neonatal outcome (3, 12). An accurate and timely immune response towards pathogens or danger signals is essential to protect against tissue damage and disease (46). However, it is equally essential that after controlling the infection

resolution of the inflammatory response is initiated by control mechanisms (28). This anti-inflammatory process is necessary to prevent further tissue damage and enable wound healing. However, the fetal immune response has been suggested to be different from the adult response with respect to this termination of inflammation. It seems that preterm infants, especially when exposed to antenatal events such as chorioamnionitis, have a limited ability to resolve the pro-inflammatory processes which promotes a status of sustained inflammation ultimately resulting in an neonatal immune system `thrown off balance`(39, 40) (Figure 3). This sustained inflammation in the neonate seems to be closely associated with an increased risk for adverse outcomes such as brain damage and pulmonary and intestinal complications (11, 41). It has indeed been shown by O`Shea et al. that increased levels of inflammation-related proteins in the blood of preterm infants measured 14 days after birth predicted severe neurodevelopmental delay at 2 years of age (49). Furthermore, it has been speculated that this sustained inflammatory response which originates in fetal life may impair neurodevelopmental processes in the long run thereby contributing to disease processes such as schizophrenia and autism (47).

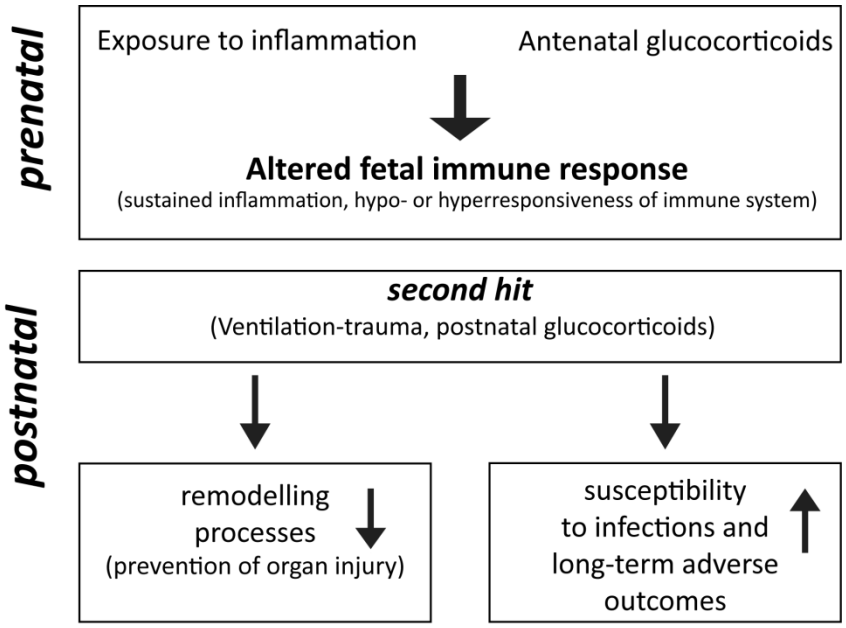

Figure 3: The fetal immune system thrown off balance. Exposure to antenatal events can affect the fetal immune response by inducing sustained inflammation or altering the responsiveness of the immune system. Additional postnatal factors can further modulate the neonate`s immunological system after birth which ultimately can result in changes in immediate tissue remodeling processes and the susceptibility for postnatal infections or long-term adverse outcomes (38).

The mechanisms by which a sustained or persistent fetal inflammatory response would increase the risk for neonatal inflammation and poor outcome remain unclear. Several explanations have been proposed: FIRS can lead to an increased risk for early postnatal bacteremia or necrotizing enterocolitis, which both would propagate the systemic inflammatory response in newborn infants (1, 58). Preterms also often need pulmonary

support via ventilation which can result in lung injury and oxygen toxicity which would further induce postnatal pulmonary and systemic inflammation (15). It is also possible that a component of the fetal inflammatory response persists, as demonstrated in **chapter 5** and **7** of this thesis by persistent activation of the fetal immunological organs, and contributes to ongoing neonatal inflammation. On a molecular level, the termination of the immune response is largely orchestrated by timely apoptosis of the activated immune cells. Phagocytosis-induced cell death (PICD) is one of the main mechanisms to remove activated monocytes thereby limiting inflammation (25). Apoptosis of these monocytes is achieved by binding of the CD95 ligand, a death ligand, to its receptor, CD95 resulting in activation of the caspase proteins which are the main effectors in cellular apoptosis pathways (31). The process of PICD seems to be diminished in cord blood monocytes of newborns due to a reduced production and secretion of this CD95L death ligand resulting in the remaining activated monocytes to be capable of producing further pro-inflammatory signals and sustaining inflammation (28).

In addition, both antenatal and postnatal events which a preterm infant encounters can further contribute to this altered neonatal immune function. It has been demonstrated that monocytes from preterm sheep function differently in respect to initiation and resolution of inflammation compared to adult cells and that their function can be further modulated by various stimuli such as endotoxin, glucocorticoids and surfactant which are all common exposures for a preterm infant (35, 36, 65). As such the complexity of exposures of the neonate to various antenatal (e.g. poly-microbial chorioamnionitis and maternal glucocorticoids) and postnatal stimuli (e.g. ventilation and postnatal glucocorticoids) influence the newborns homeostasis and makes the assessment of the neonatal immune response and consequent neonatal outcome very difficult (Figure 2).

Conclusion

Prenatal events may have a major impact on the development of the fetus *in utero*. Nearly 25 years ago, it was proposed that not only acute neonatal diseases but also adult diseases such as obesity, cardio-vascular complications, diabetes and schizophrenia are associated with exposure to prenatal factors during development, a relationship which is currently described as the Developmental Origin of Health and Disease (7, 17, 60). Understanding how these prenatal events can influence fetal development is essential in the treatment and prevention of both neonatal and adult disease processes.

The studies conducted in this PhD thesis take into account at least the two major antenatal influences, namely intra-uterine inflammation and antenatal glucocorticoids. We showed that intra-uterine exposure to inflammation can induce an immune response in the ovine fetus with inflammation and injury in the lungs, brain and immunological organs. The fetal outcome after combined exposure to maternal glucocorticoids and *in utero* inflammation

appear to be complex, depending on the timing and order of exposure. Additional mechanistic approaches are necessary to demonstrate the causal association between these antenatal exposures and changes in organ development and function in postnatal life. Furthermore, it remains to be determined what the added impact of postnatal exposures such as ventilation-trauma is on the already compromised preterm infant who was exposed to both inflammation and glucocorticoids *in utero*. Nevertheless, the findings described in this thesis have unraveled new insights into neonatal development and outcome in a relevant field of clinical application of glucocorticoids to mothers with chorioamnionitis at risk of preterm birth.

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Samenvatting

Vroeggeboorte wordt gedefinieerd door de Wereldgezondheidsorganisatie (WGO) als geboorte voor een zwangerschapsduur van 37 weken. Bijna 13% van alle kinderen worden te vroeg geboren. Ondanks de vooruitgang in de medische technologie veroorzaakt vroeggeboorte 75% van de neonatale sterfte in de Westerse Wereld. In de laatste decennia heeft de toediening van maternale glucocorticoïden en surfactant behandeling gezorgd voor een stijging van de overleving van te vroeg geborenen. Daarom wordt het nu aangeraden dat maternale glucocorticoïden worden toegediend aan vrouwen die dreigen te vroeg te bevallen. Toediening van deze antenatale glucocorticoïden stimuleren de foetale longrijping wat leidt tot een daling van het risico op sterfte, acute respiratoir falen, hersenbloedingen en darmschade bij de premature kinderen.

Chorioamnionitis is een infectie van de membranen van de placenta en vruchtwater en is één van de meest voorkomende oorzaken van vroeggeboorte. Wanneer chorioamnionitis aanwezig is, wordt de foetus blootgesteld aan de micro-organismen of pro-inflammatoire cytokines door direct contact met het besmette vruchtwater of indirect via de placentale-foetale circulatie. Blootstelling van de foetale huid, darmen en longen kan leiden tot een systemische inflammatoire response wat geassocieerd is met negatieve gevolgen voor de foetale longen, hersenen, ogen, hart, darmen, lever, thymus en immuun systeem. Aangezien dat bijna alle foetale organen kunnen aangetast worden door blootstelling aan inflammatie in de baarmoeder, is het concept van chorioamnionitis als een multi-orgaan ziekte van de foetus ontstaan.

Doordat de behandeling met maternale glucocorticoïden standaard praktijk is geworden bij een dreigende vroeggeboorte en aangezien dat de moeder bij chorioamnionitis vaak geen klinische symptomen vertoont, is de dubbele blootstelling aan zowel antenatale glucocorticoïden als chorioamnionitis veelvoorkomend bij premature kinderen. Het is echter niet duidelijk wat het effect van deze antenatale glucocorticoïden zijn op de klinische uitkomsten van de neonaat wanneer chorioamnionitis aanwezig is. Het doel van deze thesis was daarom om de effecten van gecombineerde blootstelling aan inflammatie in de baarmoeder en antenatale glucocorticoïden te bepalen op de foetale longen, immuun systeem en hersenen.

In **hoofdstuk 2, 3** en **4** hebben we het effect van blootstelling aan inflammatie op foetale long inflammatie, rijping en ontwikkeling bestudeerd. Inflammatie in de baarmoeder veroorzaakte long inflammatie en long rijping tot 14 dagen na de initiële blootstelling aan de inflammatoire prikkel. **In hoofdstuk 3** en **4** hebben we verder aangetoond dat blootstelling aan deze inflammatie de long ontwikkelings cascades Sonic Hedgehog en Wingless-Int signalering veranderde hetgeen de basis kan vormen voor verstoring van verdere foetale long ontwikkeling.

Hoewel de systemische inflammatoire respons van de foetus na inflammatie al duidelijk gekarakteriseerd is, is er weinig bekend over de reactie van de foetale immunologische organen. Daarom hebben we in **hoofdstuk 5** en **6** de respons van de foetale thymus na

blootstelling aan inflammatie onderzocht. In **hoofdstuk 7** hebben we verder aangetoond dat niet alleen de thymus maar ook de foetale milt een immuun respons kan opwekken na blootstelling aan inflammatie. Blootstelling aan een inflammatoire prikkel in de baarmoeder veroorzaakte veranderingen in het cytokine profiel en T-cel compositie van zowel de milt als de thymus tot 15 dagen na de toediening van de prikkel. Deze langdurige veranderingen in de immunologische organen tonen aan dat een bacteriële component een verlengde immuun activatie in de foetus kan veroorzaken. Dit kan mogelijk leiden tot een verstoring van de T-cel ontwikkeling en functie in het postnatale leven. Naast de negatieve effecten voor de foetale long ontwikkeling, kan antenatale inflammatie ook een effect hebben op de foetale hersenen. In **hoofdstuk 8** hebben we aangetoond dat antenatale inflammatie een immuun reactie en schade in de foetale hersenen veroorzaakt met een stijging van de microglia activatie en hypo-myelinisatie, die karakteristiek zijn voor witte stof schade in premature kinderen.

Aangezien dat er een duidelijk gebrek is aan kennis over de interactieve effecten van maternale glucocorticoïden in de context van chorioamnionitis, hebben we ook het effect van een gecombineerde blootstelling aan beide antenatale factoren op de foetale longen (**hoofdstuk 2,3 en 4**), immuun systeem (**hoofdstuk 6**) en hersenen (**hoofdstuk 8**) onderzocht. Maternale toediening van glucocorticoïden 7 dagen na de blootstelling aan de inflammatoire prikkel had geen effect op de immunologische reactie van de foetus. Tekenen van inflammatie waren aantoonbaar in de foetale longen (**hoofdstuk 2**), thymus (**hoofdstuk 6**) en hersenen (**hoofdstuk 8**) wat resulteerde in structurele veranderingen in de longen (**hoofdstuk 3**) en schade in de hersenen. Wanneer de glucocorticoïden echter werden toegediend 7 dagen voor de inflammatoire prikkel, konden er amper tekenen van inflammatie gezien worden in de onderzochte organen. Antenatale toediening van glucocorticoïden voor de inflammatoire stimulus onderdrukte de foetale inflammatoire reactie dat suggereert dat maternale glucocorticoïden het foetale immuun systeem mogelijk kunnen pre-conditioneren of verlammen. Hoewel deze situatie de onmiddellijke weefselschade voor de foetus vermindert en zo het risico op negatieve klinische uitkomsten, zoals cerebrale witte stof schade en BPD, verlaagd, kan dit toch leiden tot een hogere gevoeligheid voor postnatale infecties.

Concluderend hebben de resultaten in deze thesis aangetoond dat de timing van toediening van glucocorticoïden in de context van inflammatie in de baarmoeder de uitkomst voor de foetus bepaalt. Glucocorticoïden voor de inflammatoire prikkel weerhield de meeste van de negatieve effecten van inflammatie. Glucocorticoïden na de inflammatie aan de andere hand had enkel positieve effecten op de uitkomsten voor de foetale longen. In beide scenario's konden we echter geen extra weefselschade na gecombineerde blootstelling detecteren. Deze resultaten bevestigen daardoor de recente klinische observaties dat maternale glucocorticoïden in de context van chorioamnionitis het risico op bepaalde negatieve neonatale uitkomsten kan verminderen.

List of Publications

Peer-reviewed publications

- **Kuypers E**, Collins JJ, Jellema RK, Wolfs TG, Kemp MW, Nitsos I, Pillow JJ, Polglase GR, Newnham JP, Germeraad WT, Kallapur SG, Jobe AH, Kramer BW. Ovine fetal thymus response to lipopolysaccharide-induced chorioamnionitis and antenatal corticosteroids. PLoS One 2012;7:e38257
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Curriculum Vitae

Elke Kuypers was born on September 15th 1987 in Tongeren (Belgium). She graduated from the Heillig-Hart College Lanaken in 2005 after which she started her studies in Biomedical Sciences at Hasselt University. In 2008 she received her Bachelor`s degree and enrolled into a Master program in Clinical Molecular Sciences at Hasselt University. During this period she pursued an internship in the field of developmental biology at the department of Pediatrics at Maastricht University under the supervision of Prof. Boris Kramer who participates in international research collaborations which focuses on changes in fetal development after prenatal inflammation in an unique sheep model. After successfully completing her internships, she graduated summa cum laude as a Master in Clinical Molecular Sciences in 2010.

The unique translational nature of the research she performed during her Master`s in combination with her interest in developmental biology and neuroscience led her to pursue a PhD trajectory with Prof. Kramer and Prof. Harry Steinbusch at the School of Mental Health and Neuroscience (MHeNS) at Maastricht University. During her PhD, she focused on how exposure to intra-uterine infections can affect fetal development and how these pathophysiological changes contribute to multi-organ complications after preterm birth. She successfully completed her three year PhD trajectory with over 12 co-authorships and has recently been honored with a Kootstra Talent Fellowship grant to continue her research project to study the underlying pathophysiology of perinatal insults on fetal brain development in the research group of Prof. Boris Kramer and Prof. Harry Steinbusch.

In 2012, she married Geert Massa and together they welcomed their first son into the world in July 2013.

Elke Kuypers werd geboren op 15 september 1987 in Tongeren (België). Ze studeerde af van het Heilig-Hart College te Lanaken in 2005 waarna ze aan haar studies in Biomedische Wetenschappen aan de Universiteit van Hasselt begon. In 2008 verkreeg ze haar Bachelor titel en startte ze een Master opleiding in Klinische Moleculaire Wetenschappen aan de Universiteit Hasselt. Tijdens haar opleiding, volgde ze een stage in ontwikkelingsbiologie bij de afdeling Kindergeneeskunde aan de Universiteit van Maastricht onder begeleiding van Prof. Boris Kramer, die samenwerkt met verschillende internationale onderzoeksgroepen die de veranderingen in foetale ontwikkeling na prenatale inflammatie onderzoeken in een uniek schaap model. Na het succesvol afsluiten van haar stages, behaalde ze met summa cum laude haar Master in Klinisch Moleculaire Wetenschappen in 2010.

De unieke translationele aard van het onderzoek dat ze uitvoerde tijdens haar Master opleiding in combinatie met haar interesse in ontwikkelingsbiologie en neurologie, motiveerde haar om een PhD traject te volgen onder leiding van Prof. Kramer en Prof. Harry Steinbusch in de School of Mental Health and Neuroscience (MHeNS). Tijdens haar PhD heeft ze onderzocht hoe blootstelling aan infecties in de baarmoeder de ontwikkeling van de foetus kan beïnvloeden en hoe deze schadelijke veranderingen kunnen bijdragen tot het ontstaan van multi-orgaan complicaties bij vroeggeboorte. Ze heeft haar 3 jaar durende PhD periode succesvol afgerond met meer dan 12 co-auteurschappen en is recent gehonoreerd met een Kootstra Talent Fellowship beurs om haar onderzoeksproject naar de onderliggende pathofysiologie van perinatale insulten op de foetale hersen ontwikkeling voor te zetten in de onderzoeksgroep van Prof. Boris Kramer en Prof. Harry Steinbusch.

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